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HONOURS THESIS

Biochemical Markers as Evidence of the Effects of Environmental Enrichment on Physiological Processes in Huntington's Disease Patients

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A. INTRODUCTION

Huntingtons disease (HD) is a fatal, progressive neurodegenerative disease that has long been considered one of the most serious genetic conditions of adult life. HD exhibits a prevalence of 2.71 per 100, 000 individuals worldwide (Pringsheim et al., 2012). The genetic mutation is inherited in an autosomal dominant manner, and symptoms generally onset in mid life (1993). HD is characterised by the progressive loss of neuronal cells in the brain (Reiner et al., 1988). A multitude of symptoms with varying levels of severity are observed in the HD population depending on the level of progression of the disease, beginning with subtle motor anomalies and or cognitive defects followed by increasingly severe and devastating motor, behavioural and mental deterioration, which ultimately leads to death in HD patients (Ho et al., 2003, Paulsen et al., 2001b).

2. BACKGROUND, CURRENT UNDERSTANDING & SIGNIFICANCE

2.1. Epidemiology

HD affects all populations but with variable prevalence rates across different demographic locations. The worldwide prevalence rate of HD is 2.71 per 100, 000. Europe, North America and Australia show a particularly high prevalence rate (5.7 affected individuals per 100 000). Notably, Asia displays a low prevalence of 0.4 per 100, 000 (Pringsheim et al., 2012).

2.2. Genetics

As HD exhibits autosomal dominant inheritance, the offspring of an affected individual have a 50% risk of inheriting the mutation. The mutant *huntingtin* (*Htt*) gene is responsible for the manifestation of HD (1993, Walker, 2007)). The *Htt* gene is located on chromosome 4p16.3, from base pair 3,076,407 to base pair 3,245,686 (Walker, 2007). The mutant *Htt* gene is distinguished by an expansion in exon 1 comprising an unstable cytosine-adenine-guanine (CAG) trinucleotide repeat (Fig. 1) (Spires and Hannan, 2005). Gene negative individuals possess alleles with 10-35 CAG repeats at this location whereas gene positive individuals bear CAG repeats in excess of 35. People with 36 to 39 CAG repeats demonstrate reduced penetrance and may or may not develop the signs and symptoms of HD (Rubinsztein, 2003). Individuals with intermediate alleles, in the range of 27-35 CAG repeats, do not themselves develop HD, however they are at risk of transmitting the disease to their children through genetic anticipation (Ranen et al., 1995), which occurs due to the unstable nature of the CAG repeats. As the altered *Htt* gene is passed from one generation to the next, the size of the CAG trinucleotide repeat may increase in size (Ranen et al., 1995). Interestingly the risk of expansion is more frequent in

spermatogenesis compared with oogenesis (Pearson, 2003). HD has 100% disease penetrance in those individuals who possess 40 or more CAG repeats (1993). When there are greater than 60 CAG repeats, the individual develops Juvenile HD, a severe form with disease onset in childhood or adolescence (Andrew et al., 1993). No case of HD has been diagnosed with a count less than 36 (Brinkman et al., 1997).

Figure 1: A) Depiction of Exon 1 of the *HTT* gene in a normal individual containing less than 35 CAG repeats. **B)** Depiction of Exon 1 of the *HTT* gene in an HD carrier, with greater than 35 CAG repeats.

The CAG length accounts for only 40-50% of the variation of age of onset, with environmental factors, parental age at onset, paternal versus maternal transmission, additional genetic polymorphisms and interaction between expanded CAG length and normal alleles accounting for the remaining 50-60% of the variation (Wexler et al., 2004). A recent study conducted by Aziz and colleagues in 2009, found that the normal *Htt* allele can also affect the severity of the disease (Aziz et al., 2009a). In subjects with CAG expansions in the low range, increasing the size of the normal CAG repeat correlated with more severe symptoms and pathology (Aziz et al., 2009a). Increased CAG repeats in the mutant *Htt gene* leads to an increase in the number of glutamine residues in the encoded Huntingtin protein (HTT) (Martin et al., 2008). Disease symptoms caused by the mutant HTT protein are widespread and are discussed below.

The median age of adult onset HD occurs between 35 and 50 years (Beighton and Hayden, 1981). In approximately 10% of patients who express the juvenile variant of the disease, symptoms are evident before the age of 20 (Conneally, 1984). HD symptoms are multi factorial in nature and encompass progressive emotional, psychiatric, cognitive, psychological and motor dysfunction (Ho et al., 2003, Paulsen et al., 2001b, Soliveri et al., 2002, Smith et al., 2000).

Progressive weight loss, skeletal muscle wasting, altered sexual behaviour and sleep disturbances, in particular changes to the sleep-wake cycle, are evident in the initial stages of the disease, and are thought to be a result of hypothalamic dysfunction (Politis et al., 2008). This is further evidenced by a study conducted by Wood and colleagues in 2008 who found an increase in thirst and drinking in mice affected with HD (Wood et al., 2008). Weight loss in HD is not so much due to increased or hyperkinetic movements but is directly correlated to CAG repeat length and is likely to result from a basic deficit in metabolism and a hypermetabolic state (Aziz et al., 2008, Goodman et al., 2008). Similarly, mouse models of HD with a larger CAG repeat length have a lower body weight, despite increased caloric intake (Aziz et al., 2008).

Psychiatric problems in HD are well described and include depression, anxiety and aggression (Craufurd et al., 2001). Depression, a common psychiatric feature of HD, is often associated with increased suicidal ideation (Chatterjee et al., 2005). Mood fluctuations and anxiety disorders are also typical psychiatric features of HD (Pflanz et al., 1991, Marder et al., 2000, Paulsen et al., 2001a, van Duijn et al., 2007). Elevated levels of anxiety can also be attributed to increasing motor abnormalities that require hospitalisation (van Duijn et al., 2007).

Dementia or gradual impairment of the mental processes which are involved in comprehension, reasoning, judgement and memory are evident, together with deficiencies in attention, concentration, problem solving, executive functioning, psychomotor skills, verbal fluency and working memory deficits in HD individuals (Marder et al., 2000, Roos, 2010, Anderson and Marder, 2001, van Duijn et al., 2007).

As the level of disease progresses, the number and severity of symptoms increases. Progressive severe skeletal muscle atrophy is a common feature, possibly due to a hypermetabolic state as a result of mitochondrial dysfunction (Saft et al., 2005). Eventually patients exhibit signs of chorea, gross/fine motor skill execution impairments, as well as severe gait and balance difficulties (Cummins et al., 2011, Paulsen et al., 2008). These defects commonly lead to falls, loss of functional independence (Roos, 2010) and nursing home placement (Wheelock et al., 2003).

Patients become increasingly dysarthric, and eventually lose the capacity for effective verbal communication (Roos, 2010). Musculature involved in swallowing is also affected; aspiration pneumonia secondary to food inhalation is the predominant cause of death in HD patients (Heemskerk and Roos, 2012).

2.4. Neurophysiological features of HD

Although the HTT protein is ubiquitously expressed throughout the body, CNS degeneration is the most profound clinical feature of HD. Within the brain, the striatal neurons are most severely affected and undergo extensive degeneration (Trottier et al., 1995). Interestingly, varying levels of degeneration are observed within the different neurons in the striatum and it has been shown that 95% of GABAergic medium sized spiny neurons preferentially degenerate in HD, whereas the medium aspiny cholinergic interneurons containing somatostatin, neuropeptide Y, or nitric oxide synthase are relatively exempt from degeneration (Vonsattel et al., 1985, DiFiglia et al., 1997). Furthermore, preferential loss of striatal projection neurons has been observed at differing stages of HD progression. In the early and middle stages of HD, enkephalin-containing neurons projecting to the external portion of the globus pallidus are the most effected neurons (Zuccato et al., 2010). At the more advanced stages of the disease, projections to all striatal target areas are depleted, with the exception of the striatal projection to the substantia nigra pars compacta (Reiner et al., 1988).

One hypothesis for the increased, specific loss of striatal neurons is the observed loss of BDNF in this region. This will be described in more detail in section 3.1 of this thesis.

Although the striatum is the most profoundly affected region in the brain, studies have found that the cerebral cortex (Rosas et al., 2008, Aylward et al., 1998), globus pallidus, thalamus, subthalamic nucleus, substantia nigra, white matter (Bohanna et al., 2011, Reading et al., 2005) and the cerebellum are also markedly affected (Kloppel et al., 2009). Furthermore, the hypothalamus has been found to be significantly atrophied, and this is further evidenced by the loss of orexin, hypocretin and somatostatin-positive neurons in the lateral hypothalamus (Petersen et al., 2005, Reiner et al., 1988, Gabery et al., 2010, Soneson et al., 2010).

2.5. HTT protein

The function of the normal HTT protein is still under investigation, however studies have reported HTT to be fundamental in embryogenesis, early neural development, and craniofacial development. HTT has also been shown to be of vital importance in adult life (Reiner et al., 2003).

2.5.1 HTT in early embryonic development

It has been reported that complete deletion of the *Htt* gene in homozygous knockout mice (*Hdh*^{-/-}), results in embryonic death between day 8.5 and day 10.5 (Duyao et al., 1995, Nasir et al., 1995, Zeitlin et al., 1995). Several studies conclude that this is due to increased apoptosis in the embryonic ectoderm, shortly after the onset of gastrulation (Leavitt et al., 2001, Van Raamsdonk et al., 2005). Mice

homozygous for the expanded CAG alleles, that do not express wild type (WT) HTT protein, develop normally and have no apparent defects which suggests that the CAG repeat does not affect the physiological function of HTT during prenatal development and that mutant HTT can compensate for the loss of WT function during prenatal development (Reiner et al., 2003).

2.5.2 HTT in early neural development

HTT has been found to be critical for cortical and striatal development in mice. Late term embryos and post partum mice with HTT below 50% of WT levels, have severe defects of the cerebral cortex and striatum, telencephalic ventricular enlargement, and agenesis of fibre tracts (White et al., 1997). In more severely affected mice, brain defects are accompanied by craniofacial deformities. These developmental abnormalities tend to result in perinatal lethality. In contrast, HTT is not required for differentiation and survival of other neurons that therefore function normally. For example HTT ^{-/-} neurons in the hypothalamus, striatum, cerebellar granule cell layer and hindbrain develop normal morphology and neurotransmitter content with no evident signs of brain pathology (Reiner et al., 2001).

2.5.3 HTT in adult life

Various studies have highlighted the continued significance of normal HTT function in adulthood (Cattaneo et al., 2005, Reiner et al., 1988). They have found that HTT is essential for survival of neurons in the cerebral cortex and striatum (Reiner et al., 1988). HTT has an anti-apoptotic function. Moreover, HTT is required for the secretion of one of the most active members of the neurotrophin family of growth factors, Brain Derived Neurotrophic Factor (BDNF). The importance of HTT in relation to BDNF will be explained later in this thesis.

Studies involving immortalised striatal-derived cells overexpressing the WT HTT protein have demonstrated that the normal HTT protein is neuroprotective in CNS cells exposed to various toxic stimuli (Van Raamsdonk et al., 2005). Overexpression of normal HTT protein has also been shown to protect against ischemic injury *in vivo* in a dosage dependant manner (Leavitt et al., 2012). In contrast, cells are made more susceptible to ischemic injury by the mutant HTT protein (Zeron et al., 2002).

WT HTT protein has also been shown to be of importance in fast axonal trafficking (Gunawardena et al., 2003). Moreover, it has been postulated that HTT is an essential scaffolding protein involved in axonal transport and may have a role in initiating bidirectional trafficking (Block-Galarza et al., 1997, Gunawardena et al., 2003). Striatal neurons derived from embryonic mice which express one copy of the WT HTT allele have mitochondria that become progressively immobilised (Trushina et al., 2004).

It is important to note that the functions of WT HTT protein are still not fully understood. Insight into the structure of the protein provides some understanding of its function and therefore of the pathophysiology of HD.

2.5.4 Structure of HTT protein

The HTT protein is a large, complex, completely soluble protein, comprised of 3,144 amino acids (Zuccato et al., 2010). A notable feature of the HTT protein is the polyQ tract at its NH₂ terminus (Fig. 2) which, in unaffected individuals, contains up to 35 glutamine residues, whereas HD patients possess greater than 35 residues (Rubinsztein, 2003). The polyQ tract is a key regulator of the binding of HTT to its various interactors (Tartari et al., 2008). The polyQ region is followed by a polyproline (polyP) stretch whose function is thought to stabilise the polyQ tract by maintaining its solubility (Steffan et al., 2004).

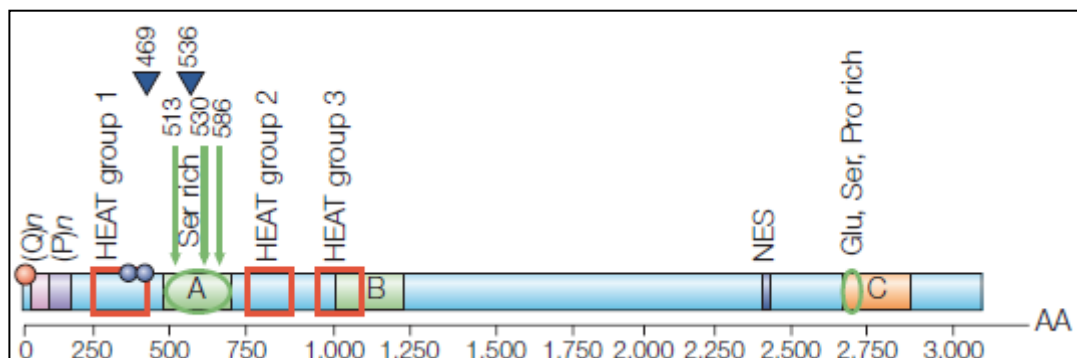


Figure 2: The HTT protein contains a polyglutamine tract (Q)_n, followed by a polyproline stretch (P)_n and 16 HEAT repeats which are arranged into 3 groups (red boxes). The HTT protein also contains a functionally active carboxy (C)-terminal nuclear export signal sequence. Adapted from Cattaneo et al., 2005.

Downstream of the polyQ tract, there are 16 HEAT repeats arranged into 3 groups. HEAT sequences are ~ 40 amino acids long and are involved in protein-protein interactions (Neuwald and Hirano, 2000).

HTT contains at least 4 cleavage sites for several proteolytic enzymes, including caspases, calpain and aspartyl proteases which cleave the protein and generate a wide range of fragments (Wellington et al., 1998). Interestingly it has been found that cleavage by caspase 6 is particularly toxic and is a crucial event in HD pathogenesis (Graham et al., 2006). The contribution of HTT proteolysis to cell functioning is unclear, however in HD, proteolysis of the mutant form of HTT causes a number of gain of function mechanisms in cells (Hermel et al., 2004) which will be explained later in this thesis.

The first 17 NH₂ terminal amino acids of HTT form an amphipathic α -helical membrane binding domain that is necessary and sufficient for its colocalisation with the golgi apparatus and the endoplasmic reticulum (Atwal and Truant, 2008, Rockabrand et al., 2007).

A functionally active carboxyl C-terminal nuclear export signal (NES) sequence and a less active nuclear localisation signal (NLS) are present in HTT, which might indicate that the protein (or a portion of it) is involved in transporting molecules from the nucleus to the cytoplasm (Xia et al., 2003). Removal of these amino acids causes the HTT to accumulate in the nucleus, as found in HD (Cornett et al., 2005).

2.6. Mutant HTT

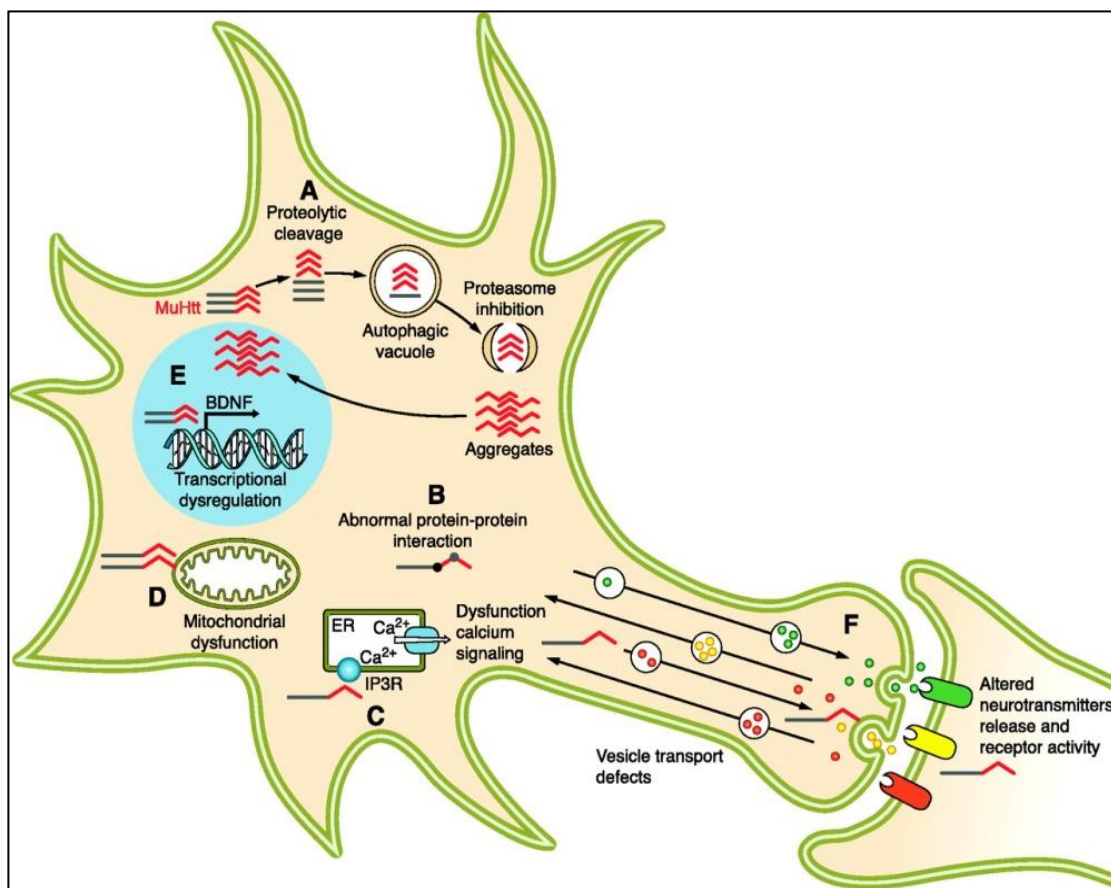


Figure 3: Representation of the various cellular pathways that are affected in the pathogenesis of HD. (A) Mutant HTT protein is more susceptible to proteolytic cleavage at its NH₂ terminal. In the cytoplasm, the fragments are targeted to the proteasome for degradation however the proteasome becomes less efficient in HD. (B) The fragments containing the expanded polyQ stretch accumulate in the cytoplasm and interact abnormally with other proteins, (C) impair calcium signalling and homeostasis, (D) bind directly to mitochondria and cause mitochondrial dysfunction, (E) translocate to the nucleus and cause transcriptional dysregulation of certain species including BDNF, or form intranuclear inclusions and (F) alters vesicular transport (Zuccato et al., 2010).

Although there is considerable evidence that normal HTT is required for cell survival, and that a loss of function of normal HTT protein can be involved in neurodegeneration, the expanded polyQ tract in mutant HTT (mHTT) has also been associated with gain of function in the pathogenesis of HD (Zuccato et al., 2010).

Mutant HTT protein fragments generated by proteolysis are targeted to the proteasome for degradation and expulsion from the cell, however the proteasome is inhibited in HD and these fragments then form aggregates in the nucleus of neuronal and non-neuronal cells in HD (DiFiglia et al., 1997) (Fig. 3). Aggregates are thought to be formed from fragments which undergo post-translational modification and subsequently misfold leading to the formation of protein aggregates which form the fibrillar structures observed in the nucleus and cytoplasm of HD patients. These aggregates then interact abnormally with other proteins (Fig. 3B), whereas full length normal HTT is predominantly diffuse in the cytoplasm of brain cells (DiFiglia et al., 1995, Li et al., 1995, DiFiglia et al., 1997). This highlights the similarities between HD and other neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and prion disorders (Ross, 2004). Protein aggregates are seen increasingly as the disease state progresses. However the presence of these protein aggregates is not necessary for neuronal death to occur (Tydlacka et al., 2008) and it is still not known if the aggregates are part of the pathogenesis of HD or an end product of an upstream toxic event. Some studies have even suggested that aggregates are actually neuroprotective because they stimulate the autophagic process and clearance of mHTT (Saudou et al., 1998).

Mutant HTT proteins bind directly to mitochondria and alter their metabolic activity and motility, and this binding increases with patient age (Fig. 3(D)) (Choo et al., 2004, Orr et al., 2008). Energy metabolism defects and oxidative stress are observed in HD (Panov et al., 2002). Moreover, mutant HTT causes cytosolic overload of Ca^{2+} by enhancing *N*-methyl-D-aspartate receptor (NMDAR) function (Fig. 3(C)) (Young et al., 1988). Finally, mHTT protein alters the vesicular transport of proteins, including BDNF (Fig. 3(F)), which will be detailed later.

2.7. Pharmacotherapies

Therapeutic targets which act upon both mutant and WT HTT protein are therefore not feasible options in the treatment of HD as HTT is fundamental for human brain development, maturation and survival. To date, no cure for HD exists and current pharmacological therapies act merely to alleviate the severity of psychiatric, cognitive and motor symptoms observed in HD, and often lack long term effectiveness (Agrawal et al., 2005). HD patients are administered a multitude of drug therapies to manage the triad of symptoms associated with HD (Agrawal et al., 2005). Current pharmacological therapies have not been

designed specifically for HD patients, but rather are designed for the general management of the symptoms observed in HD and unfortunately have a large range of undesirable side effects (Table 1) (Bonelli and Hofmann, 2004). These drugs include dopamine depleting agents, benzodiazepines, skeletal muscle relaxants, botulinum toxin and anticonvulsants for symptomatic relief of the motor impairments in HD (Ross and Tabrizi, 2011). Pharmacotherapy for the alleviation of the myriad of psychological and cognitive impairments in HD include typical neuroleptics, Selective Serotonin Reuptake Inhibitors (SSRIs), hypnotics, anticonvulsants, atypical neuroleptics and mood stabilisers such as lithium (Ross and Tabrizi, 2011). Pharmacological approaches to delay the age of onset and rate of cognitive decline in HD have not been discovered to date. There is an urgent need to identify alternate treatments capable of impacting on disease progression and improving quality of life whilst the search for a curative treatment continues.

Table 1: A summary of the drugs commonly prescribed for HD patients for the management of their symptoms and the multitude of side effects of these drugs (Ross and Tabrizi, 2011).

	Drug	Class	Main adverse effects and treatment notes
Chorea	Tetrabenazine	Dopamine-depleting agents	Depression and sedation
Myoclonus, chorea, dystonia, rigidity, spasticity	Clonazepam	Benzodiazepines	Sedation, ataxia, apathy, cognitive impairment could be exacerbated, withdrawal seizures
Myoclonus	Sodium valproate	Anticonvulsant	Gastrointestinal disturbance, weight gain, blood dyscrasia, hyperammonaemia, liver dysfunction
	Levetiracetam	Anticonvulsant	Gastrointestinal disturbance, rash, mood changes, myalgia
Rigidity (particularly associated with juvenile Huntington's disease or young adult-onset parkinsonian phenotype)	Levodopa	Amino acid precursor of dopamine	Gastrointestinal disturbance, postural hypotension, insomnia, agitation, psychiatric symptoms, increased chorea
Rigidity, spasticity	Baclofen, tizanidine	Skeletal muscle relaxants	Sedation, drowsiness, confusion, gastrointestinal disturbances, hypotension
Bruxism, dystonia	Botulinum toxin	Inhibits acetylcholine release at neuromuscular junction to cause muscle paralysis	Could paralyse nearby muscles
Psychosis, irritability	Olanzapine	Atypical neuroleptics	Sedation, parkinsonism, tardive dyskinesia, and neuroleptic malignant syndrome, but less risk of these than with older neuroleptics, raised triglycerides, weight gain from increased appetite, which could be beneficial (in relation to the weight loss seen in Huntington's disease). Caution should be exercised in patients with diabetes, and blood glucose should be monitored. Might rarely cause prolonged QT interval. Useful if patient also has agitation, irritability, and anxiety
	Quetiapine	Atypical neuroleptics	As above for olanzapine, but less metabolic syndrome
Psychosis, chorea, irritability	Risperidone	Atypical neuroleptics	As above for olanzapine, but less effect on increasing appetite
	Sulpiride	Older neuroleptics	Agitation, dystonia, akathisia, sedation, hypotension, dry mouth, constipation
	Haloperidol	Older neuroleptics	Sedation, more parkinsonism than atypical neuroleptics, dystonia, akathisia, hypotension, constipation, dry mouth, weight gain, tardive dyskinesia, higher risk of neuroleptic malignant syndrome than atypical neuroleptics
Treatment-resistant psychosis	Clozapine	Atypical neuroleptics	As for other neuroleptics, plus agranulocytosis, myocarditis, and cardiomyopathy. Needs blood monitoring
Psychosis with prominent negative symptoms	Aripiprazole	Atypical neuroleptics	Parkinsonism, akathisia, drowsiness, gastrointestinal disturbance, tremor, blurred vision
Depression, anxiety, obsessive compulsive behaviour, irritability	Citalopram	SSRI	Gastrointestinal disturbance, hypersensitivity reactions, drowsiness, syndrome of inappropriate antidiuresis, postural hypotension
	Fluoxetine	SSRI	As for citalopram, sleep disturbances
	Paroxetine	SSRI	As for other SSRIs, raised cholesterol
	Sertraline	SSRI	As for other SSRIs
	Mirtazapine	Presynaptic α_2 -antagonist, increases central noradrenaline and serotonin activity	Weight gain, oedema, sedation, headache, dizziness, tremor. Useful for sedation when insomnia is a problem
	Venlafaxine	Serotonin and noradrenaline reuptake inhibitor	Hypertension, gastrointestinal disturbance, hypersensitivity reactions, drowsiness, agitation, syndrome of inappropriate antidiuresis, palpitations
Altered sleep-wake cycle	Zopiclone, zolpidem	Hypnotics	Drowsiness, confusion, memory disturbance, gastrointestinal disturbance
Mania or hypomania	Sodium valproate	Anticonvulsants	As above for myoclonus
	Carbamazepine	Anticonvulsants	Hypersensitivity reactions, drowsiness, blood dyscrasia, hepatitis, hyponatraemia, dizziness, gastrointestinal disturbance
	Lithium	Mood stabiliser	Renal insufficiency, hypothyroidism, and tremor, with a narrow therapeutic window, and overdose can cause delirium and renal failure

3. ENVIRONMENTAL ENRICHMENT

3.1 Environmental Enrichment in Huntington's Disease

Environmental enrichment refers to specific modifications in cognitive, sensory, motor and behavioural stimuli in addition to increased physical activity and social stimulation. Environmental enrichment has been shown to be one approach that has proven successful in delaying the age of onset and symptom progression of several neurodegenerative disorders including Alzheimer's disease and Parkinson's disease (Young et al., 1999, Nithianantharajah and Hannan, 2006).

Various studies involving mouse models of HD have demonstrated that HD mice subjected to environmental enrichment, exhibit delayed disease onset, improvements in motor symptoms, slower rates of disease progression, reduced cerebral volume degeneration and a longer life expectancy. The first study of this kind was conducted by van Dellen and colleagues (2000), who provided an enriched environment to 30 HD mice which included cardboard, paper and plastic objects in the cages. By contrast a non-enriched group were housed in standardised caging. This study found a statistically significant difference in the age of onset of HD symptoms and a reduction in cerebral volume degeneration in the enriched mice compared to the non-enriched mice. A subsequent study conducted by Hockly and colleagues (2002) which investigated three levels of enrichment in HD mice (no enrichment, minimal enrichment and high enrichment); Found that enriched mice displayed a significant improvement in grip strength, in addition to a significant delay in peri-striatal cerebral volume loss. The amelioration of cerebral volume loss in both of these studies indicates a possible neuroprotective capacity of environmental enrichment in HD. In addition, a study conducted by Spires and colleagues (2004) has demonstrated that BDNF deficits in HD mice were completely rescued subsequent to environmental enrichment. BDNF is a fundamentally important neurotrophin with many functions, one of which is the maintenance of the hippocampal, cerebro-cortical and striatal neurons. Furthermore, a study conducted by Lazic and colleagues (2006) has demonstrated improved hippocampal neurogenesis, survival and integration into circuitry in HD mice after environmental enrichment. Moreover, environmental enrichment in HD mice results in significant motor and behavioural improvements compared to mice that were kept in a non-enriched environment (Schilling et al., 2004). Finally, longitudinal studies of the effects of environment enrichment demonstrated a longer life expectancy in the HD environmentally enriched mice relative to the mice that were kept in standard caging (Schilling et al., 2004).

Despite these encouraging results in transgenic mouse models of HD, very few human trials of environmental enrichment have been implemented in HD patients. Where they have been implemented, it has been found that it leads to increased self awareness and self esteem, and improved physical,

mental and social functioning, even in the latter stages of disease progression (Sullivan et al., 2001, Wexler et al., 2004). A study conducted in 2001 by Sullivan and colleagues, in 20 mid to late stage HD patients subjected to a 12 week enrichment program, resulted in improved physical, social and psychological wellbeing. Increased self-worth, social interaction and greater communication and verbalisation were also observed in these patients after treatment (Sullivan et al., 2001). A more comprehensive study assessed the effectiveness of an environmental enrichment program on forty HD patients (Zinzi et al., 2007). Subjects were required to perform 3 weeks of intensive rehabilitation, 3 times per year over a 2 year period with 11 of the 40 HD subjects completing all 6 admission periods. Rehabilitative exercises included respiratory exercise, speech therapy, occupational therapy, cognitive and physical therapy exercises. Improving motor performance was a primary aim of the study, so physical therapy included cycling, walking, stepping, balancing, resistance training and body weight transfer exercises. Upon completion, HD participants showed statistically significant improvements in motor performance ($p < 0.001$) by the Physical Performance Test and Tinetti Mobility assessment measures. Additionally the study documented improved Zung depression scores and improved psychological wellbeing (Zinzi et al., 2007). The largest and most comprehensive study of enrichment in HD patients was conducted by an international, interdisciplinary team who documented the extent and progression of the disease in a group of Venezuelan HD kindreds and noticed that prolonged environmental modifiers play a role in modifying the age of onset in HD gene positive individuals (Wexler et al., 2004).

Several studies in which exercise rehabilitation alone has been implemented in HD patients, have revealed significant improvement in symptoms (Quinn and Raio, 2002, Zinzi et al., 2007, Peacock, 1987). A study in which 10 HD patients were administered a 12 week home based exercise program showed improved flexibility, coordination and breath volume in patients after completion of the program (Peacock, 1987). A similar study which implemented a 14 week home rehabilitation program on a male HD patient resulted in improvements in his Berg Balance Score by 9 points and in functional measures of walking and speech, with improvements in dystonia, chorea and bradykinesia, and a reduction in the number of falls (Quinn and Raio, 2002). This study suggested that physical therapy in HD could improve function and the observed reduction in falls could significantly delay nursing home placement.

Taken together, these results present environmental enrichment or physical exercise as a promising therapeutic strategy to delay onset, and ameliorate symptoms in HD patients. Environmental enrichment differs from currently prescribed pharmacotherapies in that it could potentially provide substantial global benefits while doing no harm. This study will quantitatively assess the capacity of environmental

enrichment to effect global physiological changes in HD patients, using specific biomarkers present in peripheral blood and saliva. These biomarkers have been chosen as they are indicative of pathways and physiological processes known to be affected in HD.

4. BRAIN-DERIVED NEUROTROPHIC FACTOR

4.1. BDNF in HD

BDNF is an abundant member of the neurotrophin family of proteins. BDNF acts in a paracrine and autocrine manner to control a variety of brain processes including the growth, development, differentiation and maintenance of neuronal systems, neuronal plasticity, synaptic activity and neurotransmitter mediated activities (Zuccato et al., 2011). These effects are mediated by the binding of BDNF to its specific receptors such as tyrosine receptor kinase B (TrKb) and p75 neurotrophin receptor (p75^{NTR}) (Altar et al., 1997). In the brain, BDNF is active in the hippocampus, cortex, and striatum, areas vital to learning, memory, higher thinking, movement and control (Zuccato et al., 2011). BDNF itself is important for long-term memory, and plays an important role in enhancing synaptic plasticity in the brain, particularly during learning and memory, and is thus involved in preventing cognitive decline throughout life (Zuccato et al., 2011).

Normally, the striatum of the brain is known to be rich in BDNF protein yet low in BDNF-mRNA. Conversely, the substantia nigra and cerebral cortex have been found to be rich in BDNF mRNA (Fusco et al., 2003). BDNF is synthesised in the cortex and colocalises with HTT protein in cortical neurons that project to the striatum (Fusco et al., 2003). Although some studies have shown BDNF mRNA transcription in adult striatal neurons (Hofer et al., 1990), 95% of striatal BDNF is of cortical origin. BDNF is anterogradely transported from the cortex to the striatum in vesicles along the cortico-striatal afferents (Fig. 4) (Altar et al., 1997).

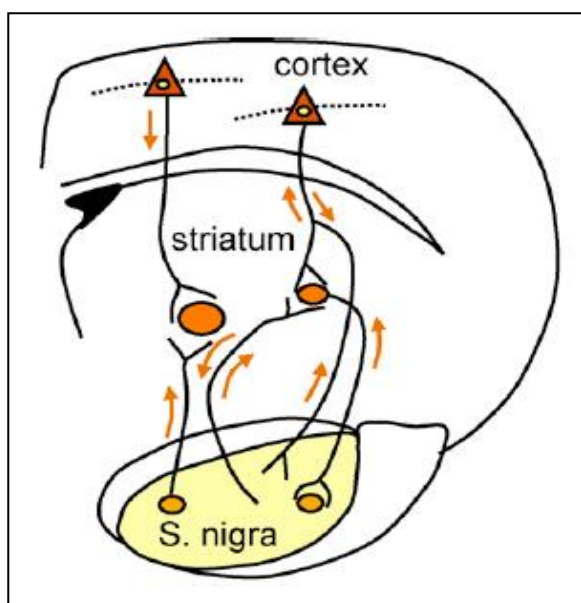


Figure 4: BDNF and the cortico-striatal synapse. The orange arrows depict BDNF anterograde and retrograde transport between the cortex and the striatum of the brain. 95% of striatal BDNF is synthesised in the cortex and anterogradely transported to the striatum. Normal HTT protein modulates the transport of BDNF along the cortico-striatal afferents and in HD this transport is dysregulated due to expression of the mutant form of the HTT protein (Zuccato et al., 2007).

BDNF deficits are most profound in the striatum of HD patients, and as detailed below, this is attributable to both the reduced cortical synthesis of BDNF and impaired transport of BDNF along the cortico-striatal afferents (Zuccato et al., 2001, Gauthier et al., 2004). Moreover, the loss of BDNF has been shown to worsen the HD phenotype and BDNF administration restores function and is neuroprotective to toxic stimuli (Gharami et al., 2008, Kells et al., 2004).

Post moretem analysis of human HD brain tissue has shown lowered BDNF mRNA and protein levels in the cerebral cortex, indicating reduced production of BDNF in HD patients (Baquet et al., 2004). Overexpression of the normal HTT protein stimulates cortical BDNF production by acting on the level of BDNF gene transcription *in vivo* and *in vitro* (Zuccato et al., 2008). Increased cortical BDNF in mice caused elevated levels of BDNF in the striatum; this effect is lost in mice that over express mHTT protein (Zuccato et al., 2008). Another study in which one BDNF allele was inactivated in a transgenic mouse line with an expanded CAG repeat, demonstrated a worsening of the HD phenotype, with an earlier age of onset and exacerbated behavioural deficits (Canals et al., 2004).

BDNF vesicular transport along the cortico-striatal afferent pathway has been shown to be significantly reduced in cells that express mHTT. HTT has been found to be part of the motor complex which drives anterograde transport of BDNF vesicles along the microtubules, and this motor complex is altered in HD (Gauthier et al., 2004). Moreover, increasing BDNF levels in the striatum confers neuroprotection against excitotoxic insult (Husson et al., 2005, Kells et al., 2008, Bemelmans et al., 1999).

Studies involving genetically engineered mice which were deficient in BDNF only in the cortex, showed that these animals gradually developed brain damage and displayed a significantly smaller

striatal volume, reduced soma size, thinner dendrites and fewer dendritic spines than WT littermates (Baquet et al., 2004). Furthermore, these mice have also been shown to exhibit striatal gene expression abnormalities which are very similar to those described in human post mortem HD striatum (Strand et al., 2007). In contrast, a study in which BDNF was constitutively overexpressed in the striatum and cortex of HD-affected mice substantially ameliorated motor dysfunction, reversed brain weight loss, restored tyrosine receptor kinase B signalling in the striatum and reduced the formation of mHTT aggregates in neurons (Gharami et al., 2008). Other studies in which BDNF was delivered via an adenoviral vector to HD-affected mice demonstrated an improvement in motor impairment, in comparison to untreated mice (Bemelmans et al., 1999, Kells et al., 2008). These results demonstrate that cortical BDNF depletion is a major contributing factor to the pathogenesis of HD and that BDNF serves as a profound therapeutic target in the treatment of HD.

4.2 Environmental Enrichment and its Effects on BDNF

Extensive studies have consistently demonstrated that environmental enrichment and voluntary exercise are associated with the transcriptional upregulation of genes encoding neurotrophins which are essential for neurogenesis, learning and memory (Rampon et al., 2000). Upregulation of BDNF expression in the cortex has been reported in response to environmental enrichment (Falkenberg et al., 1992, Pham et al., 1999, Keyvani et al., 2004). A compelling study conducted by Spires and colleagues which examined BDNF levels in HD mouse models subsequent to environmental enrichment showed that the BDNF protein deficits in the striatum and hippocampus observed in HD mice were entirely rescued after environmental enrichment (Spires et al., 2004). Furthermore, delayed onset and decline of cognitive ability has been demonstrated in mice after environmental enrichment (Pang et al., 2006). Taken together, these findings indicate that the favourable effect of environmental enrichment on neurogenesis, and neuroprotection may in part be attributed to its ability to restore BDNF levels in HD. Environmental enrichment is therefore a promising approach for delaying onset of HD and associated cognitive and physical decline. Confirmation of these results in human HD models are urgently needed however, in order for this approach to be integrated into current HD therapies.

Because BDNF is able to cross the blood brain barrier and studies carried out in rodents have shown a tight correlation between brain and blood BDNF values (Karege et al., 2002), it has been suggested that blood BDNF levels in humans may reflect BDNF levels in the brain (Karege et al., 2002). Therefore the examination of whole blood samples for levels of BDNF before and after environmental enrichment would be useful in investigating if these results are replicated in human HD patients, and will provide us with a biochemical marker to illustrate the physiological effects of environmental enrichment in HD patients.

5. CORTISOL

5.1. Cortisol in Huntington's Disease

Cortisol is a glucocorticoid that is synthesised from cholesterol in the zona fasciculata of the adrenal gland in response to stress and low levels of blood glucocorticoids (Gallagher et al., 1973). Synthesis of cortisol is regulated by corticotrophin-releasing hormone (CRH) released by the hypothalamus, which stimulates production of adrenocorticotrophic hormone (ACTH) from the anterior lobe of the pituitary gland, which in turn stimulates the adrenal cortex. When blood cortisol levels are restored, the release of CRH is inhibited (Parker, 1991). The main functions of cortisol are to increase blood sugar levels through glycogenolysis and reduction of glucose uptake into cells, suppression of the immune system, and to aid in fat, protein and carbohydrate metabolism (Gallagher et al., 1973).

Neurodegenerative disorders including Alzheimer's and Parkinson's disease are associated with neuroendocrine disturbances, and reflect changes in the hypothalamic-pituitary- adrenal (HPA) axis (Wulff et al., 2010).

Neuroendocrine cell loss and hypothalamic atrophy are evident in HD (Petersen and Bjorkqvist, 2006, Aziz et al., 2007) and studies in mouse models of HD have reported progressive alterations in the hypothalamic-pituitary-adrenal (HPA) axis (Bjorkqvist et al., 2006). Furthermore, these mice have shown progressive increases in cortisol levels which are accompanied by a Cushing-like syndrome (Bjorkqvist et al., 2006). Numerous studies have also reported elevated cortisol levels in HD patients (Leblhuber et al., 1995, Heuser et al., 1991, Bruyn et al., 1972). Cortisol levels have been correlated with disease progression in HD patients and elevated cortisol levels may be relevant to mood changes and some of the cognitive deficits that occur in HD (Aziz et al., 2009a).

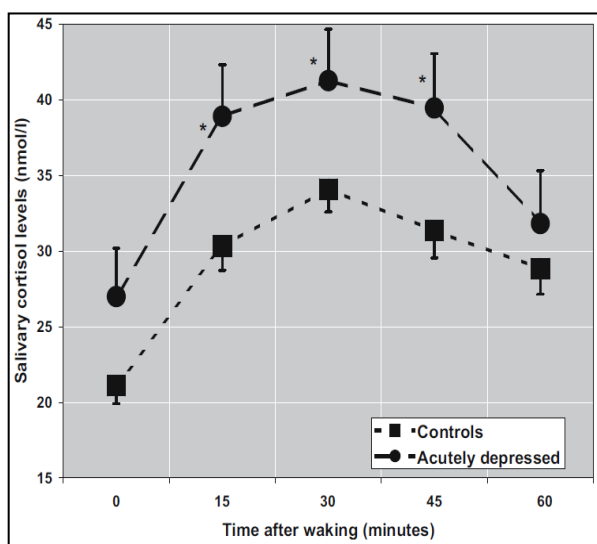


Figure 5: Morning salivary cortisol levels in relation to waking in acutely depressed subjects (n=20) and controls (n=40). Values are given as mean±SEM. *Patients have significantly increased levels of salivary cortisol at 15 min (t=-2.21, p=0.036), 30 min (t=-2.22, p=0.030), and 45 min (t=-2.36, p=0.022) compared with controls (Bhagwagar et al., 2005).

Chronic stress and depression observed in HD patients may also contribute to alteration in the HPA axis. It has been widely reported that depression and anxiety are associated with activation of the HPA axis, which is measured by the plasma concentrations of cortisol (Marashi et al., 2003, Clausturat et al., 1984, Halbreich et al., 1985). Abnormal activity of the HPA axis has been implicated as a feature of anxiety and depressive disorders, and the association between elevated cortisol and depression has been well replicated (Fig. 5) (Harris et al., 2000, Bhagwagar et al., 2005). Therefore the elevated levels of cortisol seen in HD could be attributed to either a gain of function of mHTT in HD whereby mHTT could be directly affecting the hypothalamus or pituitary gland. Alternatively or in addition to this, increased cortisol levels in HD may be a secondary effect of depression and anxiety.

5.2. Environmental Enrichment and its effects on Cortisol

There is widespread evidence that environmental enrichment causes a transient spike in cortisol levels which may be in response to the stress experienced by subjects after completing a cognitive or physical test (Robertson et al., 1981, Christensen and Galbo, 1983, Gleeson et al., 2011). The long term effects of environmental enrichment on cortisol levels however, are yet to be studied.

Physical exercise has been demonstrated to alleviate depression and anxiety (McDonald and Hogdon, 1991, Craft and Landers, 1998, Salmon, 2001). We expect these results will be replicated in HD patients after environmental enrichment. We hypothesise therefore, that environmental enrichment will partially restore the normal function of the HPA axis, which we will measure from salivary cortisol levels, and we predict that salivary cortisol levels will normalise in response to environmental enrichment. A slight improvement in depressive symptoms has already been observed in patients after a nine month environmental enrichment study (Fig. 6) and we hypothesise that this trend will continue with a prolonged environmental enrichment program of eighteen months. Evidence of these changes were assessed by analysis of salivary cortisol levels in patients.

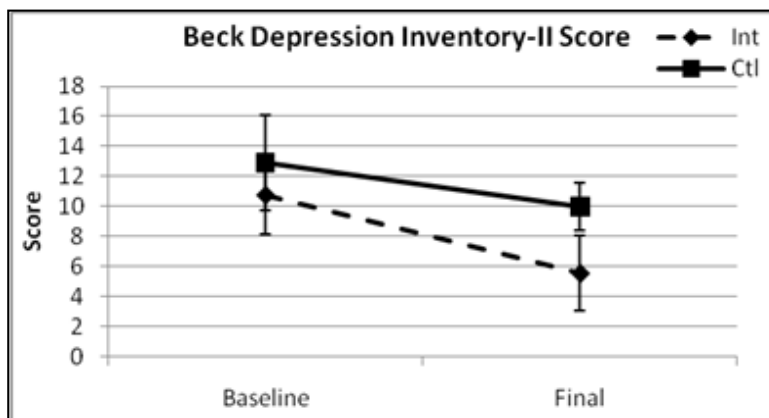


Figure 6: Beck Depression Inventory- II Scores of HD patients treated with environmental enrichment treatment (Int) versus HD patients without intervention (Ctl) at baseline and 9 months after baseline. (Thompson et al., 2012).

6. INSULIN

6.1. Insulin in HD

Insulin is a peptide hormone 51 amino acids in length that is synthesised in the beta cells of the pancreas. Insulin is secreted within the body at a constant level to remove excess glucose from the blood, which otherwise would become toxic. A constant state of hyperglycaemia is referred to as Diabetes Mellitus (Charles, 1967).

Several studies have confirmed insulin abnormalities and a higher prevalence of diabetes in HD patients. A study conducted by Podolsky and Leopold showed that 50% of HD patients (n= 14) had impaired glucose tolerance after an oral glucose tolerance test (1977). A larger study conducted by Farrer surveyed 620 HD patients and found that 10.5% had diabetes, which exceeded the age matched population prevalence (1985). Furthermore, a decrease in insulin sensitivity with an increase in insulin resistance has been found in HD patients (Lalic et al., 2008). Low insulin gene expression in the pancreas of HD mouse models has also been demonstrated (Andreassen et al., 2002).

Insulin is transported into the CNS across the blood-brain barrier by an insulin receptor-mediated process (Banks et al., 1997b, Banks et al., 1997a). Increasing peripheral insulin concentrations acutely increases the concentration in the brain; conversely, prolonged peripheral hypoinsulinaemia reduces insulin transport into the brain and downregulates blood-brain insulin receptors (Wallum et al., 1987). Insulin receptors, although abundant in the brain, are selectively distributed in the cerebral cortex, hippocampus, hypothalamus, amygdala, substantia nigra, basal ganglia and frontal cortex (Unger et al., 1991). Insulin has been reported to influence memory (Singh et al., 1997), and studies have found that intranasal administration of insulin increases memory performance in humans (Fehm et al., 2000). Therefore hypoinsulinaemia may be a contributing factor to the memory loss observed in HD patients.

6.2. Environmental enrichment and its effects on Insulin

Physical exercise has been described as being fundamental to the management of diabetes (Sigal et al., 2004). Improvement in glucose homeostasis and attenuation of insulin requirements in diabetic patients after physical exercise is a well replicated finding (Tessier et al., 2000, Boule et al., 2001, Helmrigh et al., 1991). An increase in insulin sensitivity in exercised patients has been demonstrated, possibly mediated by the upregulation of insulin receptors in these individuals (Pederson, 1980). The effect of physical exercise on insulin is yet to be studied in HD patients. As insulin levels are dysregulated in HD, as explained above, we aim to investigate insulin levels pre and post environmental enrichment and we predict that the hypoinsulinaemia which may be observed in HD patients will be attenuated upon completion of an environmental enrichment program.

In summary, it is clear that biochemical markers analysed from peripheral blood and saliva are likely to provide an estimate of the global features of HD and the effect of environmental enrichment on physiological parameters.

7. Hypothesis

Environmental enrichment globally affects physiological processes in patients with Huntington's disease as evidenced by changes in biochemical markers representative of neural and neuroendocrine pathways, and metabolic processes.

Aims

1. To investigate changes in peripheral BDNF levels in HD patients after an environmental enrichment treatment, relative to HD controls.
2. To investigate changes to peripheral blood insulin levels in HD patients after environmental enrichment treatment relative to HD controls.
3. To investigate changes in the HPA axis, identified by changes in salivary cortisol levels in HD patients after environmental enrichment treatment, relative to HD controls.

8. Theoretical Framework

Environmental enrichment has been demonstrated to prolong age of onset and alleviate symptoms in mouse models of HD. Physical exercise and short trials of environmental enrichment in HD patients have also demonstrated effective modulation of symptoms. An 18 month trial of environmental enrichment was completed at ECU by Thompson et al. (in press) in HD patients. This is the longest trial of continual environmental enrichment treatment in HD to date. The physical, neurological and cognitive changes as a result of this study were measured and results indicated amelioration of motor, physical and cognitive symptoms. In the project described here, we investigated the physiological effects of environmental enrichment in a quantitative manner, using biochemical markers to deduce whether environmental enrichment has global physiological effects on patients.

Neural dysfunction in HD is well documented and is thought to occur in part as a result of BDNF depletion. Physical exercise upregulates BDNF, therefore we will investigate whether BDNF losses in HD can be ameliorated by environmental enrichment. Similarly the HPA axis has been observed to be dysfunctional in HD and the secretion of cortisol, a marker of the HPA axis, is dysregulated in HD. Physical exercise has been shown to restore HPA function. Here we assessed cortisol levels after environmental enrichment treatment as a marker of HPA function in our patients, relative to HD

controls. Metabolic dysfunction is widely reported in HD and there have been some reports of impaired glucose tolerance in the HD population, indicating dysregulation of the insulin pathway. Physical exercise enhances insulin sensitivity by upregulating insulin receptors, therefore we will assess whether insulin levels in HD can be amended by an environmental enrichment treatment program.

9. METHODS

9.1. Research Design and Methodology

Twenty five symptomatic HD patients were recruited by Dr Jennifer Thompson of the HD group at Edith Cowan University, in collaboration with the Neurosciences Unit North Metropolitan Area Mental Health Service (NMAMHS). Ethical approval for the study of HD patients before, during and after an eighteen month environmental enrichment program has been granted by the Human Research Ethics Committees of Edith Cowan University and the North Metropolitan Area Mental Health Service. Patients provided informed consent to participate in the study.

9.1.2. Participants

Potential participants were identified and recruited utilising NMAMHS databases. Inclusion criteria consisted of a positive genetic test, clinical disease diagnosis (UHDRS-TMS ≥ 5), ability to follow verbal or written instructions and the capacity to perform sub-maximal exercise. Exclusion criteria included recent substance abuse, an unstable psychiatric or medical condition, or confounding neurological conditions. Each patient was given a Z score for cognitive and motor ability at baseline. Patients were then assigned to two groups equally matched for cognitive and motor Z scores to reduce any confounding effects due to differences between groups. Age, gender, age at diagnosis, disease duration, CAG repeat number, CAG index and BMI differences were analysed and no significant differences ($p > 0.05$) between the two groups were found for these variables at baseline (Table 2). As these covariates were already controlled for at the beginning of the study, it is not necessary to analyse their effect on the markers used in this investigation. Participants were randomised to an intervention group (IG) or delayed intervention group (DIG) as explained below. It was not possible to blind participants to group allocation.

Table 2: Outline of covariate measures of the Intervention and Delayed Intervention Group. No significant difference was found between the two groups in relation to the variables indicated below ($p > 0.05$). (Thompson et al., 2012)

Characteristic	Intervention Group (IG) (mean \pm SEM)	Delayed Intervention Group (DIG) (mean \pm SEM)
Number of participants	9	11
Gender M/F	4/5	6/5
Age (years)	53.8 \pm 2.9	52.2 \pm 2.6
Age at Diagnosis (years)	49.5 \pm 3.2	49.7 \pm 2.5
Disease Duration (years)	4.3 \pm 1.8	2.4 \pm 0.7
CAG Number	43.1 \pm 1.1	43.7 \pm 0.7
CAG Index	399.0 \pm 53.7	416.1 \pm 23.3
Body Mass Index (kg/m ²)	26.2 \pm 1.3	27.1 \pm 1.3

9.1.3. Medication: A thorough medical history and complete list of medications was taken at the beginning of the study and at each assessment point for each patient. Participants remained on their medication throughout the study, which was adjusted where necessary by physicians. All participants were deemed fit to participate by a clinician.

9.1.4. Intervention Design

An interdisciplinary team comprised of neurologists, psychiatrists, psychologists, physiotherapists, occupational therapists, and exercise physiologists were involved in the design of the intervention which was formulated after the analysis and interpretation of baseline data. As HD is a highly variable disease, the intervention was individually tailored in order to maximise safety and efficacy. Thereafter each patient worked at their own capacity, and the intervention was progressed on an individual basis when the exercise physiologists deemed individuals physically capable. There were also gender differences in the ability of patients to perform the exercises. The implementation of a weekly aerobic and resistance training program was carried out in an exercise clinic by exercise physiologists. Physiotherapists and exercise physiologists designed and implemented a home-based program. Occupational therapists formulated personalised programs that targeted deficits detected at baseline by psychologists.

9.1.5. Intervention

The clinical gym exercise program consisted of nine months of once-weekly supervised group exercise sessions conducted in a clinic gym setting. In order to reduce potential falls and to minimise the risk of injury resistance training machines were utilised rather than free weight training. In order to maximise patient compliance, a variety of times and locations were offered to minimise travel and lifestyle constraints. Each exercise session included a 5 minute warm up period, 10 minutes of aerobic exercise, 40 minutes of resistance exercise and a 5 minute cool down period.

In addition, a personally tailored home-based exercise program including aerobic and resistance exercise components was prescribed to patients, to be performed three times weekly. This program was performed independently after precise instruction by the interdisciplinary team, and was not employed until exercise physiologists deemed that the person was physically capable of performing it safely. Personalised occupational therapy programs designed to maintain self-care, leisure and personal independence were provided to patients on a weekly basis in the home environment.

In order to facilitate the standardisation of the clinical exercise sessions, monitor adherence and track physical progression of patients, exercise physiologists were given an exercise manual to follow and a logbook to record exercise data.

To maximise safety and increase compliance of the home-based program, an exercise DVD, manual and diary were provided to all intervention participants. Exercise physiologists additionally reminded participants to perform the home-based exercise program and complete their exercise diaries.

9.1.6. Physical and Neuropsychological Measures

A clinical battery of motor, cognitive, physiological and functional assessments were used to assess patients. The UHDRS-TMS was used to assess changes in motor function. Additional outcome measures assessed functional, cognitive, depressive and quality of life changes.

Body composition was quantified using a Hologic Dual X-ray Absorptiometry Scanner (DEXA). Routine physical strength tests were used to assess changes in muscle strength. Postural stability changes were examined using the Sensory Organisation Test (Neurocom SMART Balance Master, NeuroCom). Neuropsychological tests included the Symbol Digit Modalities Test, Hopkins Verbal Learning Test-Revised, Colour Word Interference Test and Trail Making Trials. The Goal Attainment Scale examined achievement of goals. Levels of depression were assessed using the Beck Depression

Inventory-II. Finally, quality of life perceptions of patients and carers were evaluated using the SF-36v2 Health Questionnaire and Huntington's Disease Quality of Life Battery for Carers (HDQOL-C).

In the study performed for this thesis, biomarkers indicative of physiological parameters were assessed at baseline, during, and at the end of the study. The remainder of the methods and results will detail these experiments.

10. QUANTIFICATION OF BIOMARKERS

10.1.

Aim 1: Quantification of BDNF in Patient Blood

Background

Measuring BDNF from patient brain tissue samples is not possible in our study. However, BDNF is able to cross the blood brain barrier and it has been suggested that blood BDNF levels may reflect the BDNF levels in brain (Trajkovska et al., 2007). Studies carried out in rodents have shown a tight correlation between brain and blood BDNF values (Karege et al., 2002). We therefore measured BDNF from whole blood samples. Moreover, recent literature has revealed that BDNF is better assessed in whole blood than in serum when samples are stored for extended periods of time. Long term storage of serum samples (6 - 10 months) has been associated with significantly lower serum BDNF concentrations which suggest that in whole blood samples, BDNF stored in platelets is protected from degradation (Trajkovska et al., 2007).

To measure BDNF, non-fasting blood samples were obtained from patients at 0, 9 and 18 months by venipuncture of the antecubital vein and collected into EDTA blood tubes. Samples were collected from all patients over 2 days, within two weeks upon completion of the study. BDNF has a diurnal rhythm, with variation between males and females (Fig. 7) (Piccinni et al., 2008), therefore utmost care was taken to obtain samples from patients at midday (12.30- 2.00pm), when male and female BDNF levels cross over.

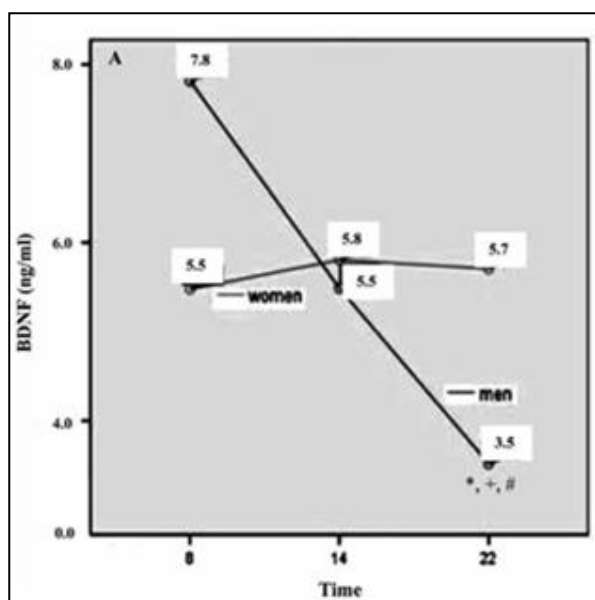


Figure 7:

Plasma BDNF levels at three time-points in both males (n= 14) and females (n=14). In males, plasma BDNF concentrations (mean+SD, ng/ml) were 7.82+5.61 at 08:00 h, 5.49+4.34 at 14:00 h, and 3.52+2.75 at 22:00 h. In females, BDNF levels were 5.48+2.26 at 08:00 h, 5.80+2.67 at 14:00 h, and 5.71+3.79 at 22:00 h. Significant diurnal variation in plasma BDNF concentration is evident in males ($p = 0.008$) (Piccinni et al., 2008).

Method

BDNF was analysed in whole blood which was taken from patients at 0, 9 and 18 months of the study. Samples were not centrifuged as this would rupture thrombocytes and release BDNF into the serum, where it may be subjected to proteolysis (Trajkovska et al., 2008). Whole blood samples were stored at -20°C until required.

When samples were ready to be measured, blood was thawed, inverted several times and 3% Triton-X100 was added to disrupt the cell membrane of the thrombocytes. The mixture was then sonicated for 10 seconds at output 3 of the sonicator, to further rupture the cell membranes. The time and intensity of sonication was determined for optimal release of BDNF into the serum, and details of this optimisation are explained in Table 7. The samples were then centrifuged to separate the plasma and the plasma was tested for BDNF using the BDNF BEK-2002-2P sandwich ELISA Kit (Biosensis, Ukraine), which was modified for use in our laboratory as follows:

The standards were prepared and 100 μL of the standards, controls and plasma samples were pipetted into the microtiter wells of a 96 well plate which were pre-coated with polyclonal human BDNF antibody. A 1:10 dilution of the plasma samples was made before samples were added to the wells. Samples, standards and controls were measured in duplicate. The plate was sealed with parafilm and left to incubate overnight at 4°C on a plate shaker. The plate was then washed five times with pre-prepared wash buffer (0.85% NaCl, 0.14% Na_2HPO_4 , 0.02% NaH_2PO_4 , $7.2 < \text{pH} < 7.6$). 1000 μL of biotinylated antibody solution was added to each well. This solution contained a detection antibody which bound to the antigen, thus completing the 'ELISA sandwich'. The plate was sealed and incubated at room temperature on a plate shaker for 3 hours. The plate was then washed five times, after which 1000 μL of

1:99 Avidin-Biotin-Peroxidase Complex (ABC) working solution diluted with ABC buffer was added to each well. The plate was sealed and incubated on a plate shaker for an hour. The plate was then washed five times with wash buffer after which 90µL of the peroxidase substrate, tetramethylbenzidine (TMB) colour developing agent was added to each well and left to incubate for 5-20 minutes. Thereafter, 100µL of TMB stop solution was added to each well to halt the reaction. The intensity of the coloured product is directly proportional to the concentration of BDNF present in the samples. The absorbance of each well was read at 450nm using a Fluostar II (BMG LAB TECH) absorbance reader within 10 minutes of the addition of stop solution. Concentrations of BDNF in patient samples were determined from the standard curve of absorbance readings of standards provided in the kit.

10.2.

Aim 2: Quantification of Insulin in Patient Blood

Background

In order to quantify insulin levels in patients, we measured C-peptide (Fig. 8) levels in fasting plasma samples from HD patients taken at 0, 9 and 18 months of the environmental enrichment program. Samples were collected from all patients over 2 days. Fasting blood samples were taken as this provides an accurate baseline measurement of insulin levels, unaffected by food ingestion.

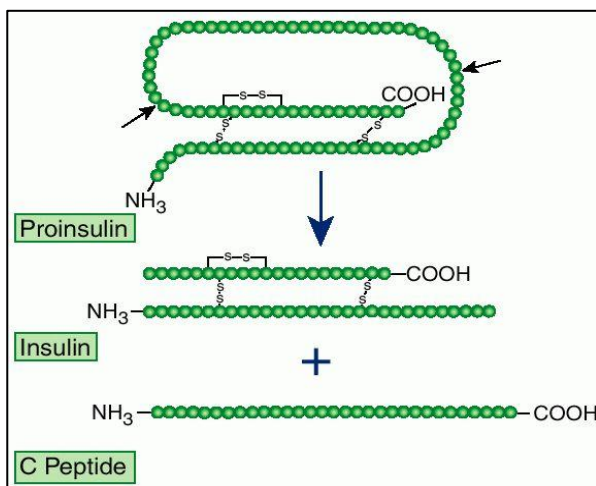


Figure 8: Insulin is synthesised in pancreatic beta cells as a component of proinsulin. Proinsulin is enzymatically cleaved releasing insulin into the circulation along with a residual fragment, Connection - peptide (C-peptide). Thus, C-peptide is present in equimolar amounts with insulin (<http://medicaldictionary.thefreedictionary.com/insulin>).

Measurements of C-peptide were taken because the half life of C-peptide is between 2 and 5 times longer than that of insulin (Horwitz et al., 1975). Furthermore, C-peptide is metabolically inert, with C-peptide levels in venous blood approximately 5-6 times greater than that of insulin levels (Beyer et al., 1979). Another advantage to using an insulin assay is that a C-peptide assay is able to distinguish endogenous insulin from injected insulin. A C-peptide ELISA kit (DE1293) (DEMEDITEC) was

utilised to measure C-peptide levels, and the methodologies described in the ELISA kit manual were adapted for our studies as follows:

Method

Patient blood was drawn from the antecubital vein by venipuncture, collected into EDTA tubes and centrifuged immediately to separate the plasma. The plasma was aliquoted and stored at -20°C until used in this project.

Thawed samples were inverted several times prior to testing. 100µL of each standard, control and sample were dispensed into a 96 well microtiter plate pre-coated with anti-mouse antibodies to C-peptide. Each standard, control and sample was measured in duplicate. Each sample was combined with 50µL of anti-serum, a monoclonal anti C-peptide antibody and 100µL of enzyme conjugate. The mixture was mixed then incubated at room temperature for 60 minutes with shaking at 400-500 rpm. The wells were then emptied and washed three times with 400µL of diluted wash solution. 100µL of enzyme complex was then added to each well and the wells were incubated for 30 minutes at room temperature with shaking at 400 rpm. The wells were then washed again as described above and 100µL of substrate solution was added to each well. This was followed by an incubation period of 20 minutes at room temperature after which the enzymatic reaction was halted by the addition of 100µL of stop solution (1 mol/L H₂SO₄). The intensity of colour observed was inversely proportional to the concentration of C-peptide in the patient sample. The absorbance of each well was determined using a Fluostar II (BMG LAB TECH) absorbance reader at 450 nm within 10 minutes of adding the stop solution. Patient concentrations of C-peptide were derived from the standard curve obtained from the absorbance readings of the standards provided in the kit.

10.3.

Aim 3: Quantification of Cortisol in Patient Saliva

Background

Cortisol was measured from saliva samples taken at six specific times during the day over 3 consecutive days to capture the diurnal rhythm (Fig. 9), with levels peaking in the early morning and dropping to the lowest values at night (Hucklebridge et al., 2005). Levels of cortisol rise independently of circadian rhythm in response to stress (Miller et al., 2007).

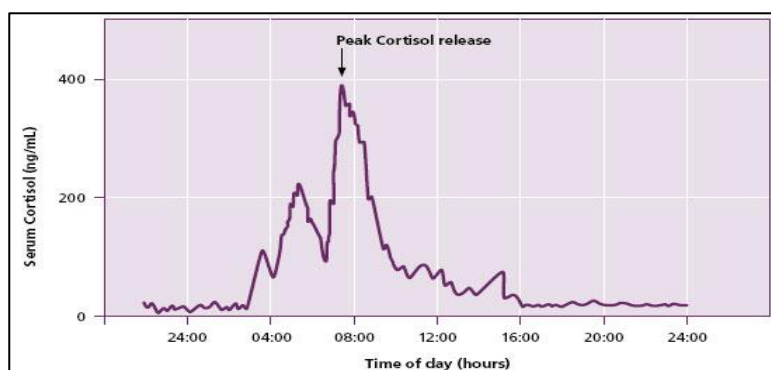


Figure 9: Cortisol production has a diurnal rhythm with levels peaking early in the morning and dropping to their lowest values at night (Abudu, 2009).

Salivary cortisol levels were measured in our project because studies have consistently reported a high correlation between salivary and serum cortisol levels (Dorn et al., 2007), which indicates that salivary cortisol levels are a reliable measure of serum cortisol levels. Moreover, salivary cortisol levels are not affected by salivary flow rate and are relatively resistant to degradation from enzymes or freeze thaw cycles (Vining and McGinley, 1987, Garde and Hansen, 2005). Blood samplings require mildly invasive techniques that may cause stress and thereby alter HPA axis activity (Stahl and Dorner, 1982), additionally, cortisol in blood is largely bound to plasma proteins (Angeli et al., 1978) whereas salivary cortisol has been shown to be correlated with plasma free cortisol, the biologically active fraction (Katz and Shannon, 1969). Due to the diurnal rhythm of cortisol secretion, we assessed cortisol levels at six time points. For this reason it was also more practical to assay cortisol in saliva rather than blood samples.

Method

Saliva samples were taken from patients using Salivette tubes (SARSTEDT) over three consecutive days at the times specified in Table 3. As cortisol levels have been reported to have the greatest variability overnight between HD patients (van Duijn et al., 2010), morning and evening cortisol levels were averaged from two overnight samples.

Table 3: Outline of the various times saliva samples were taken from patients. Two overnight readings were taken as it has been speculated that overnight readings are the most variable amongst HD patients.

Day	Time
1	20:00 hrs
2	08:00 hrs 12:00 hrs (before consumption of lunch) 16:00 hrs 20:00 hrs
3	08:00 hrs

Saliva samples were taken at 0, 9 and 18 months of the environmental enrichment program. Food or drinks, with the exception of water were not consumed by the patients 1 hour prior to taking the saliva sample and smoking was not allowed for the 30 minutes prior to providing the sample. Patients refrained from consuming alcohol 12 hours prior to commencement of measurement of saliva samples. Salivette tubes containing saliva samples were frozen at -20°C by patients.

In this study, salivary cortisol levels were quantified by using a High Sensitivity Salivary Cortisol ELISA KIT 1-3002 (SALIMETRICS). The protocol was adapted in our study as follows:

All reagents and saliva samples were brought to room temperature before use. Saliva samples were first vortexed for 10 seconds before they were centrifuged for 15 minutes at 3000 rpm. A 1X wash buffer was then prepared from the 10X wash buffer included in the kit. Duplicates of standards, controls and samples were pipetted into the wells of a 96 well microtiter plate provided in the ELISA kit, pre-coated with monoclonal antibodies to cortisol. Cortisol enzyme conjugate (cortisol linked to horseradish peroxidase) was then added to each well, followed by 60 minutes of incubation on a plate shaker. The wells of the plate were then washed four times with wash buffer, to wash away any unbound components, before the addition of TMB substrate solution to measure the bound cortisol peroxidase. The plate was shaken for 5 minutes and then incubated for 25 minutes in the dark. After incubation, 50µL of stop solution was added to each well, the plate was then shaken for 3 minutes again. The absorbance of each well was read at 450nm using a Fluostar II (BMG LAB TECH) absorbance reader within 10 minutes of the addition of stop solution. The amount of cortisol peroxidase was measured by the intensity of colour which was inversely proportional to the amount of cortisol present. A standard

curve was prepared from the absorbance readings of standards provided in the kit, and concentrations of cortisol in patient saliva samples were estimated from the standard curve.

11. DATA ANALYSIS

The concentrations of the above biomarkers were obtained at baseline, 9 months and 18 months after intervention. The differences in levels of each biomarker between baseline, 9 months and 18 months were calculated for each participant. These values were then averaged to obtain a mean and standard error of the mean (SEM) for each group over each different time period. Results were analysed using a one way ANOVA to assess statistical significance (Fig. 10). A p value ≤ 0.05 was deemed statistically significant. Below is a schematic detailing the timeline of the intervention and the analysis of the results in the 2 different groups.

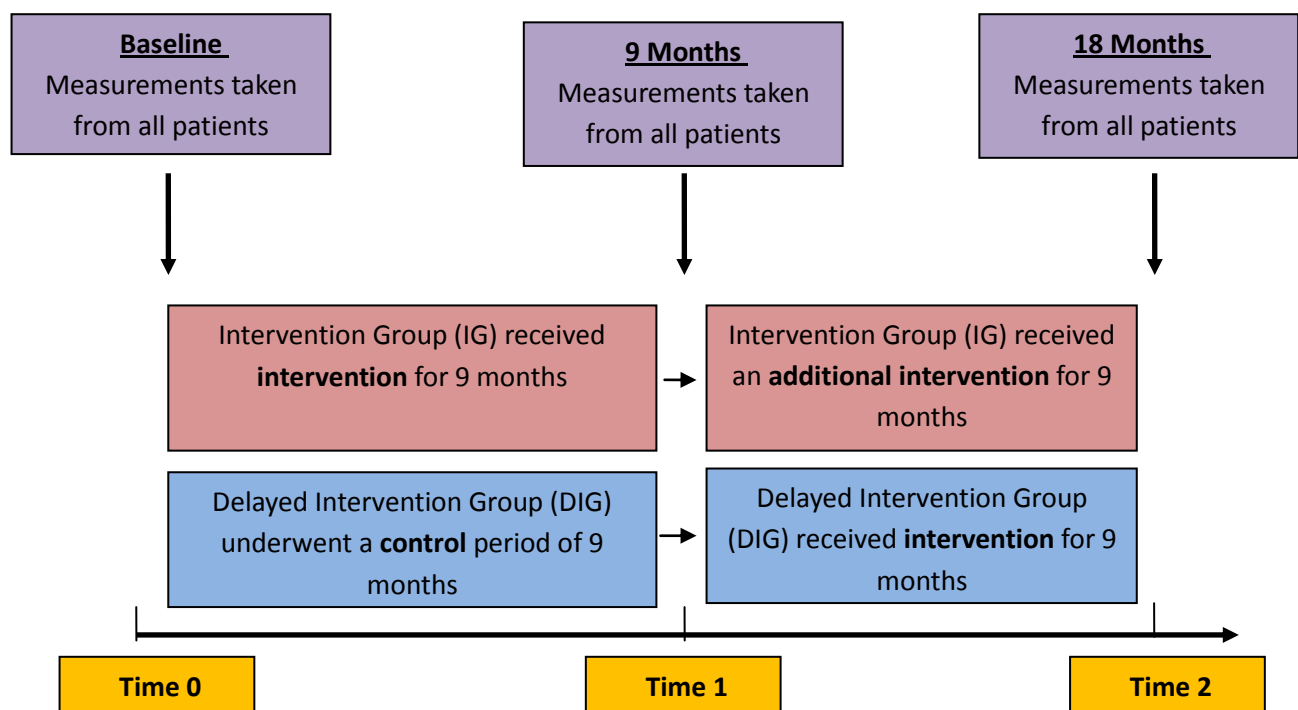


Figure 10: Schematic outlining the different participant groups in our study. The **delayed intervention group (DIG)** (blue) were tested for baseline measures at Time 0 and did not receive environmental enrichment treatment for 9 months (a control period). Thereafter, intervention was provided for 9 months from Time 1 - Time 2. The **intervention group (IG)** (red) were tested for baseline measures at the beginning of the study, Time 0, and were given 2 rounds of intervention, therefore receiving a total of 18 months of intervention with measures taken at Time 1 and at the end, Time 2. By using this study design we are able to study the effects of intervention versus HD controls (no intervention) as well as compare the effects of a longitudinal intervention relative to a shorter intervention. We also compared results for controls (no intervention) relative to those after 18 months of intervention.

12. RESULTS

Current pharmacological therapies prescribed to HD patients act merely to manage symptoms, and do nothing to delay the onset or rate of decline of this fatal disease. These therapies are not specifically designed for HD but for the general symptoms observed in HD and have a large range of side effects (Tabrizi et al., 2011). Environmental enrichment has proven successful in delaying onset and symptom progression in mouse models of several neurodegenerative disorders including Parkinson's and Alzheimer's disease (Goldberg et al., 2012, Jeong et al., 2011, Rodriguez et al., 2011). Furthermore, several studies in mouse models of HD have demonstrated delayed onset, improvement in motor symptoms, slower rate of disease progression, amelioration of cerebral volume degeneration and behavioural improvements (Hockly et al., 2002, Lazic et al., 2006, Spires et al., 2004, van Dellen et al., 2000). In humans, short trials of environmental enrichment in HD patients have shown promising results including improved physical, social, psychological and motor function and depression scores (Sullivan et al., 2001, Zinzi et al., 2007). Taken together, environmental enrichment is a promising therapeutic strategy to delay onset and ameliorate symptoms in HD, with potential global physiological effects.

An 18 month environmental enrichment intervention was conducted by ECU researchers. This study is the longest trial of continual environmental enrichment in HD to date. In this study we investigated the effects of environmental enrichment on physiological processes which are dysregulated in HD. Here we describe analysis of changes in levels of biochemical markers as evidence of the global physiological effects of environmental enrichment on HD patients. Three biochemical markers were used to analyse these changes on physiological processes; BDNF, insulin and cortisol.

Neural dysfunction in HD is thought to occur in part due to BDNF depletion (Zuccato et al., 2001). As physical exercise upregulates BDNF (Falkenberg et al., 1992, Keyvani et al., 2004, Pham et al., 1999), we investigated whether BDNF protein levels in HD patients are affected by environmental enrichment. Metabolic dysfunction is widely reported in HD, and impaired glucose tolerance in the HD population has been widely demonstrated, indicating the dysregulation of insulin in these patients (Lalic et al., 2008). Physical exercise enhances insulin sensitivity by upregulating insulin receptors, therefore we assessed whether insulin dysregulation in HD can be amended by an environmental enrichment treatment program. Insulin was indirectly assessed by measuring levels of C-peptide (as described in section 8.2). Similarly the HPA axis has been observed to be dysfunctional in HD (Aziz et al., 2009b, Bjorkqvist et al., 2006) and the secretion of cortisol, a marker of the HPA axis, is known to be dysregulated in HD (Aziz et al., 2007, van Duijn et al., 2010). Physical exercise has been shown to

restore HPA function (Salmon, 2001). Here we assessed cortisol levels after environmental enrichment treatment as a marker of HPA function in our patients.

At the beginning of the environmental enrichment study, baseline measurements of all participants were taken, after which patients were divided into either an intervention group (IG), or a delayed intervention group (DIG). The IG, (represented in red in graphs), underwent the intervention for the first 9 months of the study. Thereafter they received a further 9 months of intervention, i.e. a total of 18 months of intervention. The DIG, (represented in blue in graphs) underwent a control period (no intervention) for the first 9 months of the study, followed by 9 months of optimised intervention (Fig. 10). The details of the intervention and the timeline for each group are described in Table 4 and Fig. 10 respectively.

Table 4: Details of the intervention provided to the DIG and IG over the 18 month study period.

	0 - 9 Months of the study (Time 0 - Time 1)	9-18 months of the study (Time 1 – Time 2)
<u>Intervention Group (IG)</u>	<p>IG received 9 months of intervention which consisted of:</p> <ul style="list-style-type: none"> • 1 x weekly supervised gym visit. • 1 x weekly self-directed home based exercise. • 1 x weekly home visit by occupational therapist. 	<p>IG received an additional 9 months of intervention (longitudinal intervention) which consisted of:</p> <ul style="list-style-type: none"> • 1 x weekly supervised gym visit. • 1 x weekly self directed home based exercises.
<u>Delayed Intervention Group (DIG)</u>	<p>DIG had no intervention:</p> <p>Control Period in which patients carried out their normal day to day activities</p>	<p>DIG received an optimised intervention which consisted of:</p> <ul style="list-style-type: none"> • 1 x weekly supervised gym visit. • 1 x weekly exercise physiologist-supervised home based exercise. • 1 x weekly home visit by occupational therapist.

12.1. BDNF Levels in Response to Environmental Enrichment:

Whole blood samples were sonicated at output 3 on the sonicator for 10 seconds as this was found to be the optimal protocol for maximal BDNF release, without causing damage to the BDNF protein (See Table 5 for summary of sonication optimisation). Samples were analysed for BDNF using the BDNF BEK-2002-2P sandwich ELISA KIT (Biosensis, Ukraine). The details of the method are described in section 8.1. Samples were run in duplicate in the same assay. Intra-assay CV was 2.4%, and the inter-assay CV was 4.45%.

Table 5: BDNF optimisation to identify the optimal time of sonication, output level and dilution factor of sample using the probe or water bath method. Blood used for sonication attempts was obtained from a healthy female.

Attempt	Aim	Conditions	Time	Dilution Factor (Whole Blood: 3% Triton X)	Outcome
1	To investigate the sonication procedure by examining a range of sonication times	Sonicator probe at Output 3	15 s, 30s, 45s and 60s (600ul sample)	1:2	<ul style="list-style-type: none"> High background reading Obtained BDNF concentrations 5 x lower than normal range Standard curve was inaccurate ($r=0.89$) 45 s blood sample coagulated after sonication
2	To investigate the use of a water bath sonicator as all samples are able to be sonicated at one time.	Water Bath sonicator at 80% output	30 s, 45 s, 60 s	1:2	<ul style="list-style-type: none"> Obtained BDNF concentrations that were 10x below normal expected range
3	To improve yield by increasing intensity and time of sonication BDNF concentrations obtained in attempt 2 were too low.	Water Bath sonicator at 100% Output	1.5 min, 2 min, 2.5 min, 5 min, 7min, 10min	1:2	<ul style="list-style-type: none"> Obtained BDNF concentrations that were 10x below normal expected range
4	To investigate probe sonicator again as water bath sonication method still yielded BDNF concentrations too low even at highest intensity for prolonged time of sonication. A more diluted sample was used as ELISA kit may not be able to detect concentrated BDNF levels	Probe sonicator at output 3	10s, 15s, 30s, & 45 s	1:2 and 1:10	<ul style="list-style-type: none"> 1:10 dilution with 10 s sonication at output 3 yielded BDNF concentrations closest to normal expected range.
5	A range of outputs and times around the 10s mark were investigated as 1:10 dilution with probe- sonication at output 3 for 10 s yielded the best result in attempt 4.	Probe sonicator at Ouput 3, 4, 5 and 6	8s, 10s, 12s	1:10	<ul style="list-style-type: none"> 1:10 dilution with probe sonication at output 3 for 10s still yielded the BDNF concentration closest to normal range (Fujimura et al., 2002), therefore these conditions were utilised in analysing patient samples.

Baseline levels of BDNF were not significantly different between the DIG and the IG ($p>0.05$) and were below the normal expected range (Fig.11a). In the DIG, a gradual decrease in BDNF levels was seen in patients over time; BDNF levels decreased during the 9 month control period and this trend was also seen during the 9 month intervention. These changes were not statistically significant ($p>0.05$).

In the IG, a significant decrease in BDNF levels was observed during the first round of intervention ($p=0.03$). Interestingly, BDNF levels increased slightly upon completion of the second round of intervention relative to levels at 9 months, but the increase was not significant ($p>0.05$). After 9 months of intervention, BDNF levels were significantly lower ($p = 0.011$) in the IG compared to the DIG which underwent a 9 month control period during this time (Fig. 11a, Table 6).

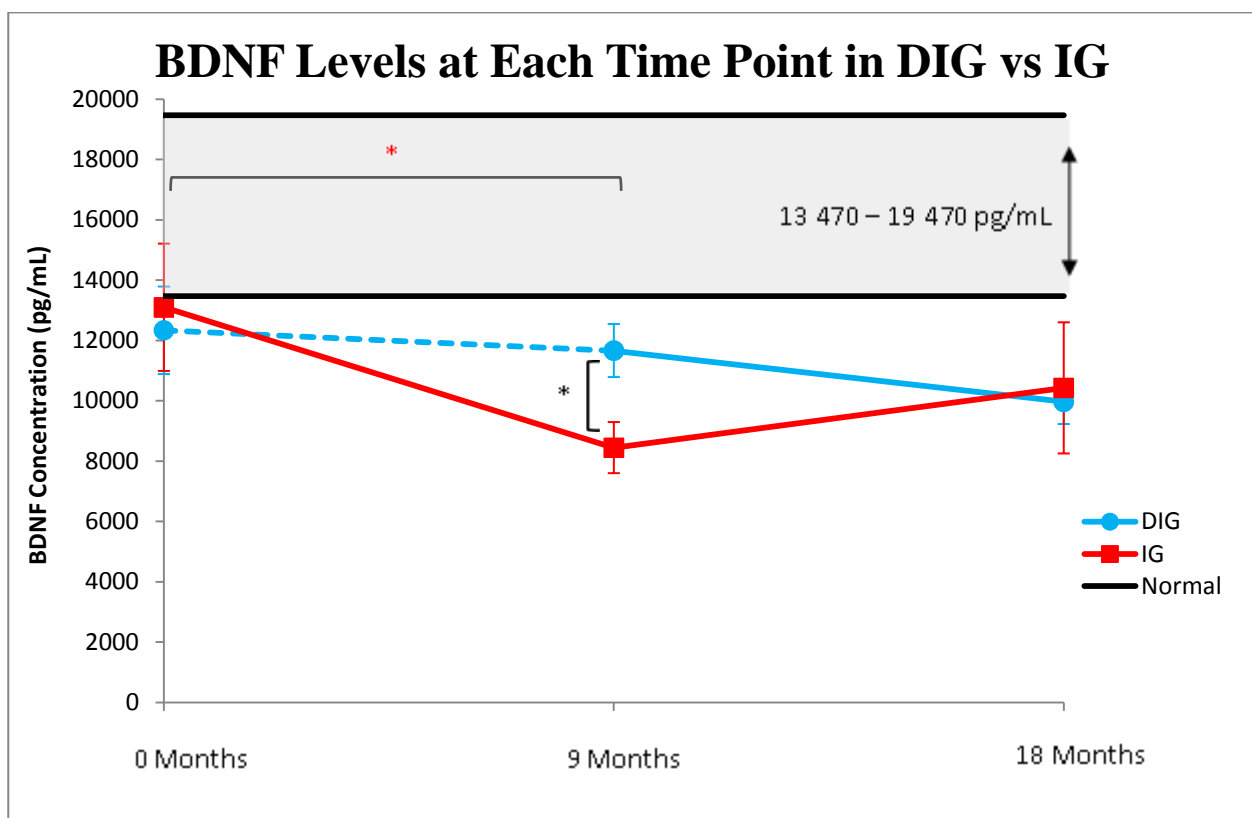


Figure 11a: BDNF concentrations from whole blood at 0 Months, 9 Months and 18 Months after the commencement of the study in the DIG (blue) and the IG (red). The blue dashed line indicates the 9 month control period in the DIG. The grey shaded area represents the normal expected range for BDNF concentrations. Values are presented as mean \pm SEM * $P < 0.05$. DIG (n) = 10 IG (n) = 8.

In order to reveal the relative changes in BDNF levels, the differences in BDNF levels between time points were calculated (Fig. 11b, Table 6). During the 9 month control period in the DIG, BDNF levels decreased slightly by an average of 816 pg/mL. Similarly, a slight decrease in BDNF levels was also seen in this group after 9 months of intervention, although these decreases were not statistically significant ($p>0.05$).

In the IG, a decrease in BDNF levels by 4 652 pg/mL was also seen after the first round of intervention. By contrast, after the 18 month longitudinal intervention in this group, a significant increase in BDNF levels was observed (p=0.03).

BDNF levels decreased from baseline by an average of $-3\,276 \pm 1184$ pg/ml after one round of 9 months of intervention in both groups.

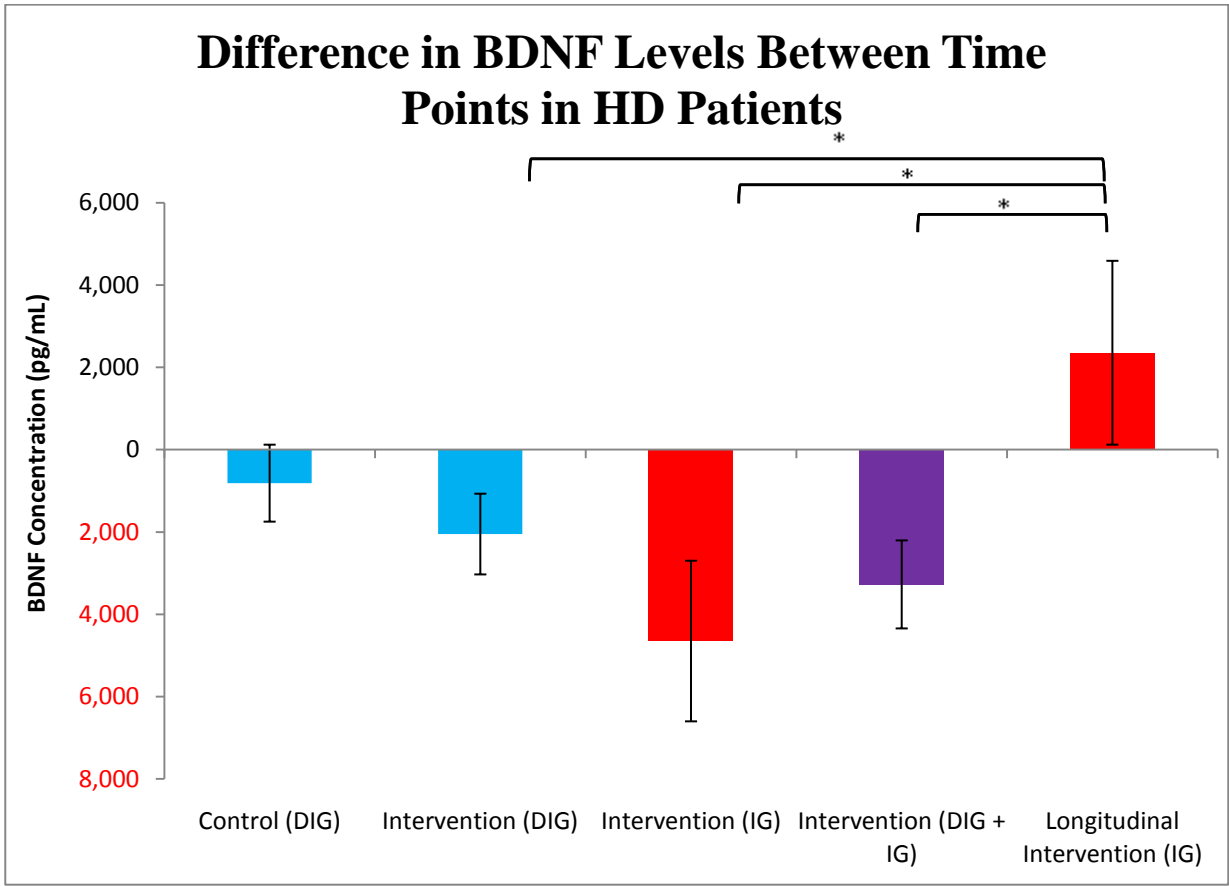


Figure 11b: The mean difference in whole-blood BDNF concentrations after a 9 month control period in the DIG (blue), 9 months of intervention in IG (red) and the DIG (blue), after 9 months of intervention in both groups was combined (purple) and after 18 months of longitudinal intervention in the IG (red). Values are presented as mean ± SEM *P < 0.05. DIG (n) = 10 IG (n) = 8.

Table 6: Mean BDNF concentrations at each time point in the DIG (blue) vs the IG (red) and P Values after 2-way Repeated Measure ANOVA analysis. Values are represented as mean \pm SEM.

	Mean BDNF Concentration (pg/mL \pm SEM)			Mean Difference Between Time Points (pg/mL \pm SEM)			P Value (2 Way Repeated Measures ANOVA)		
	Time 0	Time 1	Time 2	Time 1 – Time 0	Time 2 – Time 1	Time 2 – Time 0	Time 0 vs Time 1	Time 1 vs Time 2	Time 2 vs Time 0
IG	13 095 \pm 2 111	8 443 \pm 847	10 423 \pm 2 174	-4 652 \pm 951	2 352 \pm 2 233	-1 261 \pm 2 096	0.03	0.19	0.20
DIG	12 333 \pm 1 450	11 661 \pm 880	9 966 \pm 740	-816 \pm 935	-2 052 \pm 1 033	-2748 \pm 1219	0.41	0.08	0.27
P Value (2 Way ANOVA) IG vs DIG	0.25	0.01	0.41						

As mentioned earlier, BDNF levels vary between males and females, therefore we aimed to investigate BDNF levels in males and females separately. Both male and female BDNF levels were below the normal expected range for each gender (shown in triangles in Fig. 11c) (Trajkovska et al., 2007).

In both the IG and the DIG BDNF levels in females were observed to fluctuate particularly after either the control or intervention periods, whilst levels remained relatively stable in males throughout the study. This can be seen by the difference in patterns represented by the pink (females) and green (males) lines in Fig. 11c.

In the DIG at 0 months, and after the 9 month control period, BDNF levels were significantly higher in females compared to males ($p=0.05$ and $p=0.005$ respectively). After 9 months of intervention in this group, female BDNF levels decreased to levels similar to those seen in males (this decrease was not significant $p>0.05$), and by the end of the study at 18 months, levels did not differ between males and females ($p>0.05$).

In the IG, levels of BDNF did not differ significantly between males and females throughout the entirety of the study ($p > 0.05$). After the first 9 months of intervention in this group, BDNF levels decreased in both males and females and after 18 months of intervention BDNF levels rose sharply in females to

16 126 pg/mL, this rise however was not significant ($p>0.05$). After 18 months of longitudinal intervention in this group, males and females appeared to respond differently.

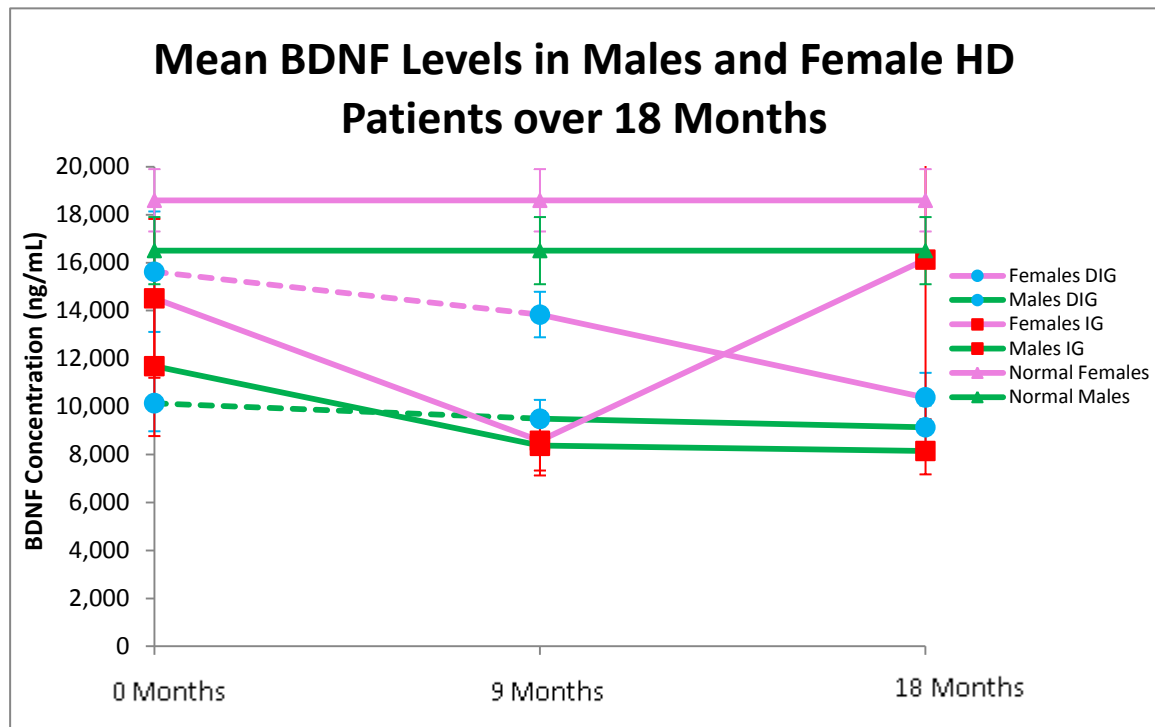


Figure 11c: Mean whole blood BDNF concentrations in males (green) and females (pink) at each time point in the study. Normal expected values for females are represented in pink (▲) and normal expected values for males are shown in green (▲). The DIG is shown in blue (●) and the IG is shown in red (■). The 9 month control period is represented by a dashed line in the DIG. Values are presented as mean \pm SEM. DIG Females (n)= 6, Males (n)= 4. IG Females (n)= 2, Males (n)= 6.

To further investigate the BDNF gender response, the difference in BDNF levels between time points was calculated separately for males and females (Fig. 11d). After the control period, BDNF decreased in both males and females in the DIG, and after 9 months of intervention in this group, BDNF levels continued to decrease in females, however increased slightly in males by an average of 750 ± 284 ng/mL. The male response was significantly different to the female response seen during the intervention in the DIG ($p=0.031$).

After 9 months of intervention in the IG, a decrease in BDNF was seen in both males and females. After an additional 9 months of intervention in this group, BDNF levels increased significantly in females ($p=0.026$), and levels remained the same in males ($p>0.05$) relative to levels at 9 months of intervention. There was a significant difference in the change in BDNF levels between males and females after the longitudinal intervention ($p=0.027$). However, the increase in BDNF after the 18 month longitudinal intervention in the IG was not significantly different to the decrease in BDNF levels seen during the 9 month control period in the DIG ($p>0.05$).

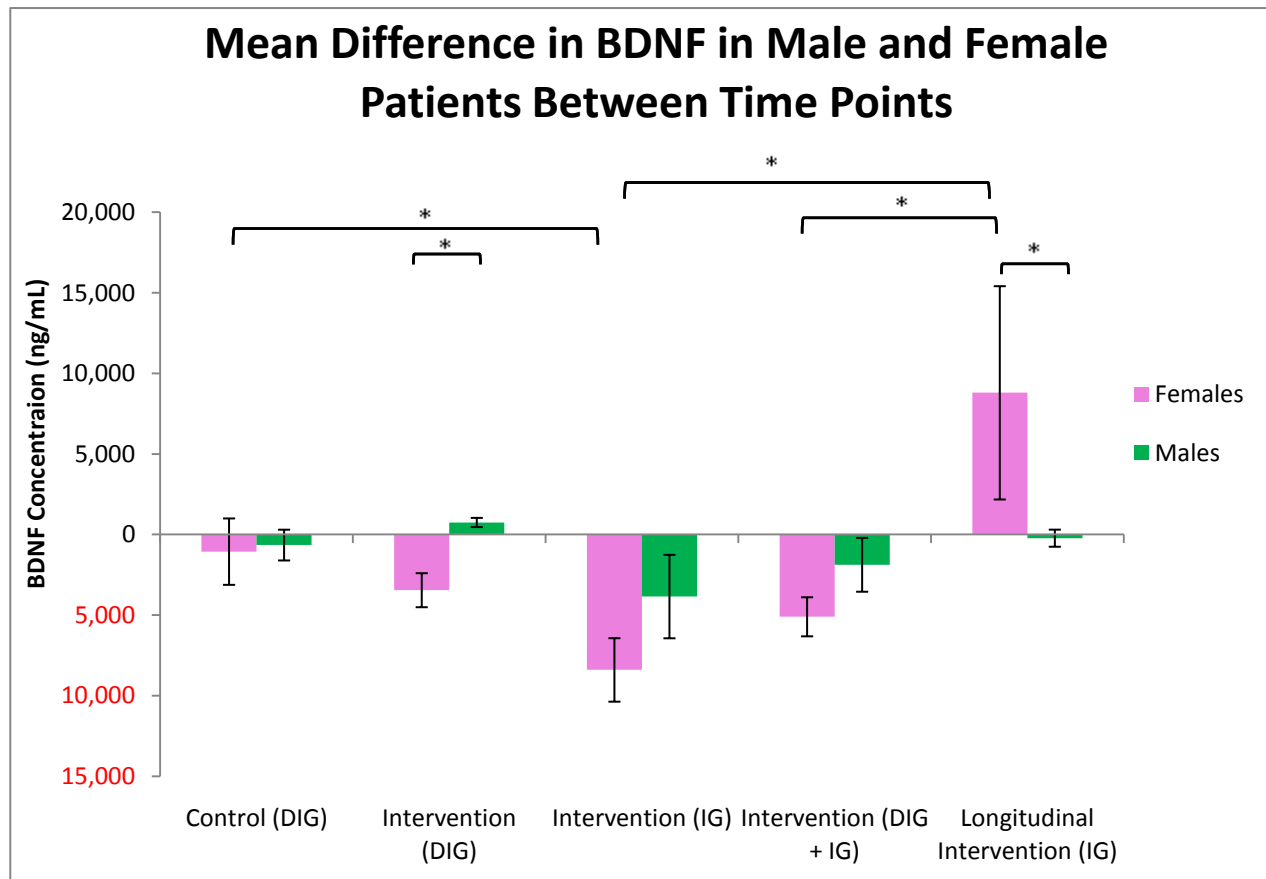


Figure 11d: The mean difference in whole-blood BDNF concentrations in males (green) vs females (pink) after a 9 month control period in the DIG, 9 months of intervention in IG and the DIG, after 9 months of intervention in both groups was combined and after 18 months of longitudinal intervention in the IG. Values are presented as mean \pm SEM *P < 0.05. DIG Females (n)= 6, Males (n)= 4. IG Females (n)= 2, Males (n)= 6.

12.2. C-peptide Measurements in Response to Environmental Enrichment:

Analysis of fasting plasma was performed using a C-peptide ELISA kit DE1293 (DEMEDITEC) as described in section 8.2. Samples were run in duplicate in the same assay. Assay results were considered acceptable only if the coefficients of variation (CV) were <5%. Intra-assay CV were < 5% (2.5%), and the inter-assay CV was <5% (4.2%).

Baseline fasting plasma C-peptide levels of all participants were within the expected range of fasting C-peptide concentrations for normal individuals (shown as grey shaded area in Fig. 11a) (Endocrine Sciences Inc.). Moreover, baseline levels of C-peptide were not significantly different between the IG (red) and the DIG (blue) ($p = 0.226$) (Fig. 12a, Table 7).

In both groups over the 18 months of the study, we observed an increase in C-peptide levels. After the first 9 months of the study, C-peptide levels were significantly increased relative to baseline regardless of whether patients received the intervention or not (control period) ($p < 0.001$). This increase resulted in C-peptide levels exceeding normal levels (Fig. 12a). During the next 9 months there was a significant increase in C-peptide levels relative to levels at Time 1 in the DIG even after 9 months of intervention ($p = 0.022$). Moreover there was a significant increase in C-peptide levels by 18 months in the IG relative to levels at 9 months ($p = 0.008$).

C-peptide levels were 3.1 fold higher than the higher end of normal levels (2.1 ng/mL) in the delayed intervention group and 2.8 fold higher than normal values in the intervention group at the end of the study.

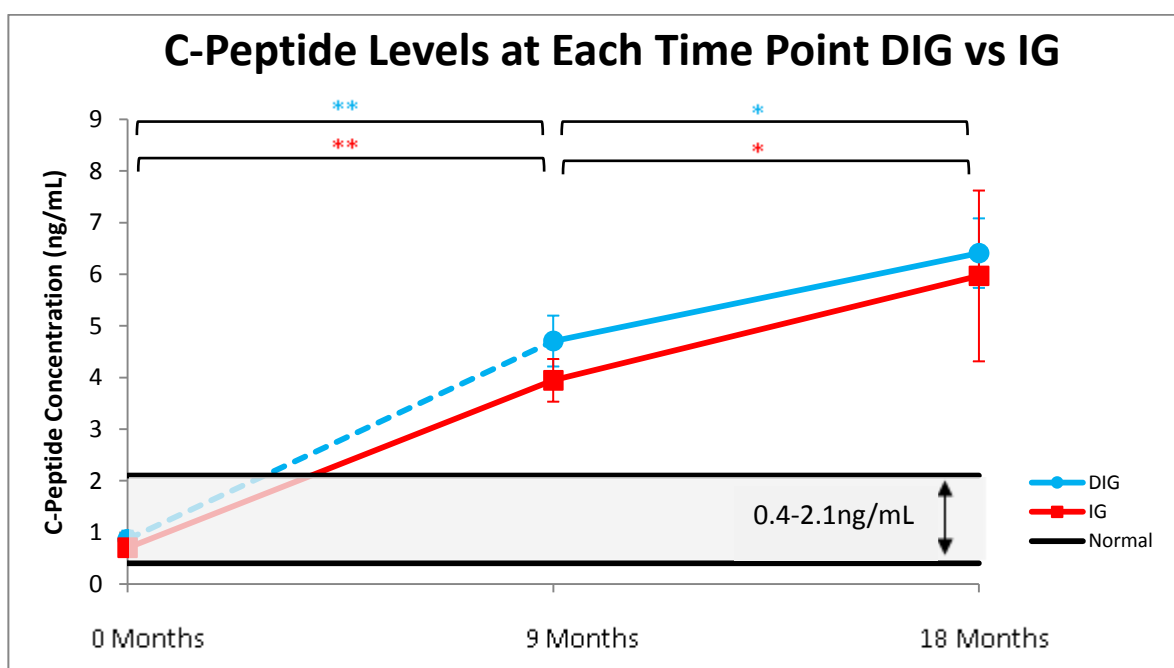


Figure 12a: Mean fasting plasma C-peptide concentration at each time point in the DIG (blue) vs the IG (red). The grey shaded area represents the normative range for fasting plasma C-peptide concentrations in healthy adults (Endocrine Sciences Inc.). The blue dashed line indicates the 9 month control period in the DIG.. Values are represented as mean \pm SEM. * $P \leq 0.05$ ** $P \leq 0.001$. DIG (n)= 10, IG (n)= 12.

Table 7: Mean fasting plasma C-Peptide concentration at each time point in the DIG (blue) vs the IG (red) and P Values after 2-way ANOVA analyses. Values are represented as mean \pm SEM.

	Mean Fasting Plasma C-peptide Concentration (ng/mL)			Mean Difference in C-peptide Levels Between Time Points (ng/mL)			P Value (2 Way Repeated Measures ANOVA)		
	Time 0	Time 1	Time 2	Time 1 – Time 0	Time 2 – Time 1	Time 2 – Time 0	Time 0 vs Time 1	Time 1 vs Time 2	Time 2 vs Time 0
IG	0.70 \pm 0.47	3.95 \pm 0.39	5.97 \pm 1.01	3.77 \pm 0.38	2.26 \pm 0.89	6.03 \pm 1.28	P<0.001	0.008	0.003
DIG	0.87 \pm 0.17	4.71 \pm 0.49	6.41 \pm 0.67	4.20 \pm 0.52	1.49 \pm 0.82	5.69 \pm 1.33	P<0.001	0.022	P<0.001
P Value (2 Way ANOVA) IG vs DIG	0.226	0.131	0.361						

To investigate the relative change in C-peptide levels within each group, we assessed the mean differences in C-peptide levels between time points. During the 9 month control period in the DIG, C-peptide levels increased by an average of 4.20 ± 0.52 ng/mL (Fig. 12b, Table 7). After a 9 month intervention in the DIG, C-peptide levels increased by 1.49 ± 0.82 ng/mL (Fig. 12b, Table 7). This increase was 2.81 fold lower than the increase seen in the control period in this group ($p=0.022$) and therefore, there was a significantly reduced increase in C-peptide levels in this group, possibly as a result of the intervention.

An average increase of 3.77 ± 0.38 ng/mL was observed in the IG during the first 9 months of their intervention (Fig. 12b, Table 7). After an additional 9 months of intervention in the IG, C-peptide levels increased by a further 2.26 ± 0.89 ng/mL (Fig. 12b, Table 7). Although, this increase was 1.7 fold lower than the increase in C-peptide levels during the first 9 months of intervention in this group, indicating that the additional enrichment continued to reduce the increase in C-peptide levels, it was not found to be statistically significant ($p>0.05$).

C-peptide levels were shown to be increased by an average of 2.56 ± 0.47 ng/mL after one round of 9 months of intervention in both groups (Purple column of Fig. 12b).

The increase in C-peptide levels after the first intervention in the IG was significantly higher than the increase in C-peptide levels after the optimised intervention in the DIG (p=0.009), indicating that the differences in the intervention received by the patients may have had an effect on C-peptide levels.

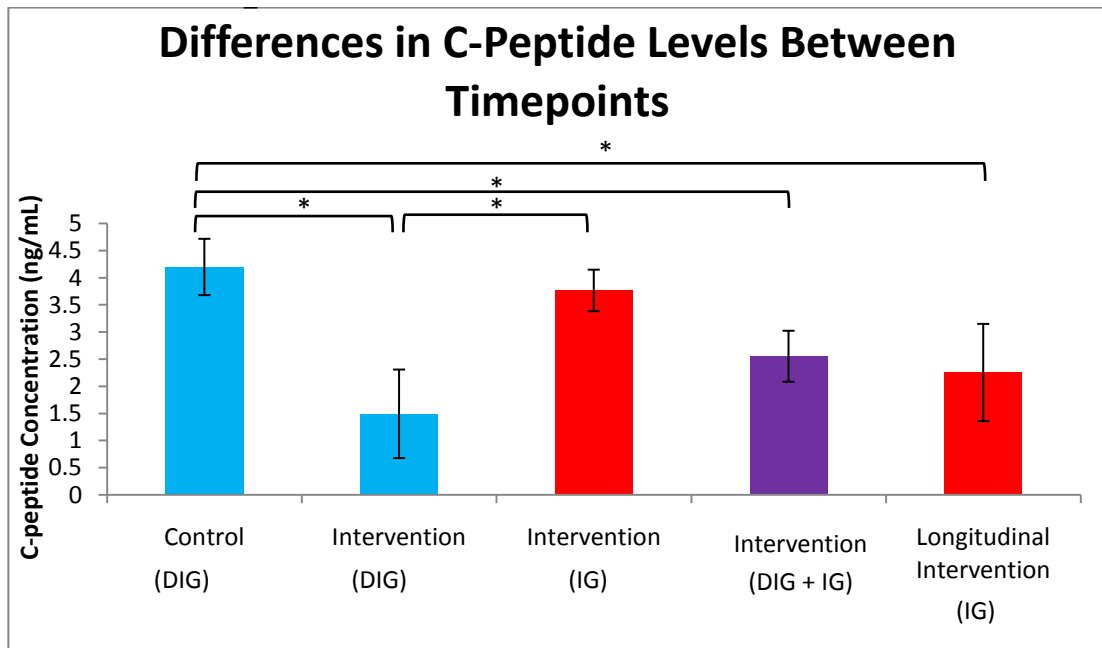


Figure 12b: The differences in C-peptide levels between time points was measured for each patient and the mean of these differences is shown after a 9 month control period in the DIG (blue), 9 months of intervention in the IG (red) and the DIG (blue), after 9 months of intervention in both groups was combined (purple) and after 18 months of longitudinal intervention in the IG relative to measurements taken after IG. Values presented are mean \pm SEM. *P < 0.05. DIG (n)= 10, IG (n)= 12.

12.3. Salivary Cortisol Levels in Response to Environmental Enrichment:

Analysis of salivary cortisol was performed using a High Sensitivity Salivary Cortisol ELISA KIT 1-3002 (SALIMETRICS), the details of the method are described in section 8.3. Samples were run in duplicate in the same assay. Intra-assay CV was 1.36%, and the inter-assay CV was 4.60%.

Salivary cortisol levels followed the typical diurnal secretion pattern (Fig. 13a and Fig. 13b) as seen in the normal population. In both the DIG and IG, levels of cortisol increased with the progression of time regardless of whether patients received the intervention or underwent a control period.

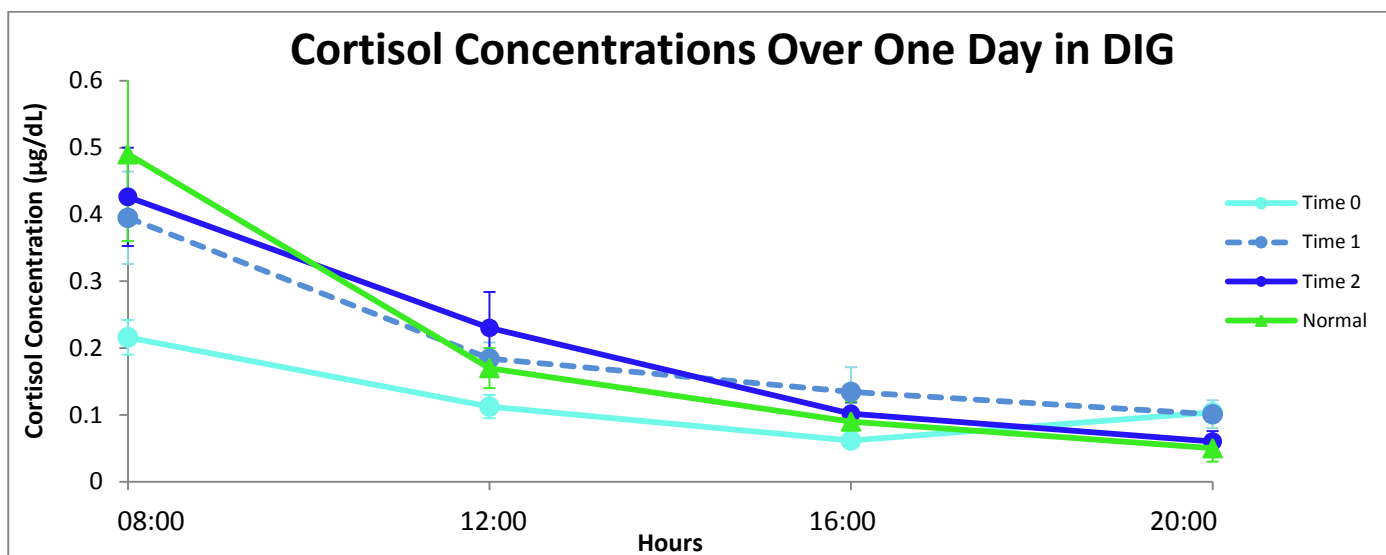


Figure 13a: Mean salivary cortisol levels of HD patients in DIG at 08:00, 12:00, 16:00 and 20:00h in DIG at Time 0 (baseline), Time 1 (after control period) and Time 2 (after intervention) vs expected levels in normal population (green). Values are presented as mean \pm SEM. * $P \leq 0.05$ (n)= 12.

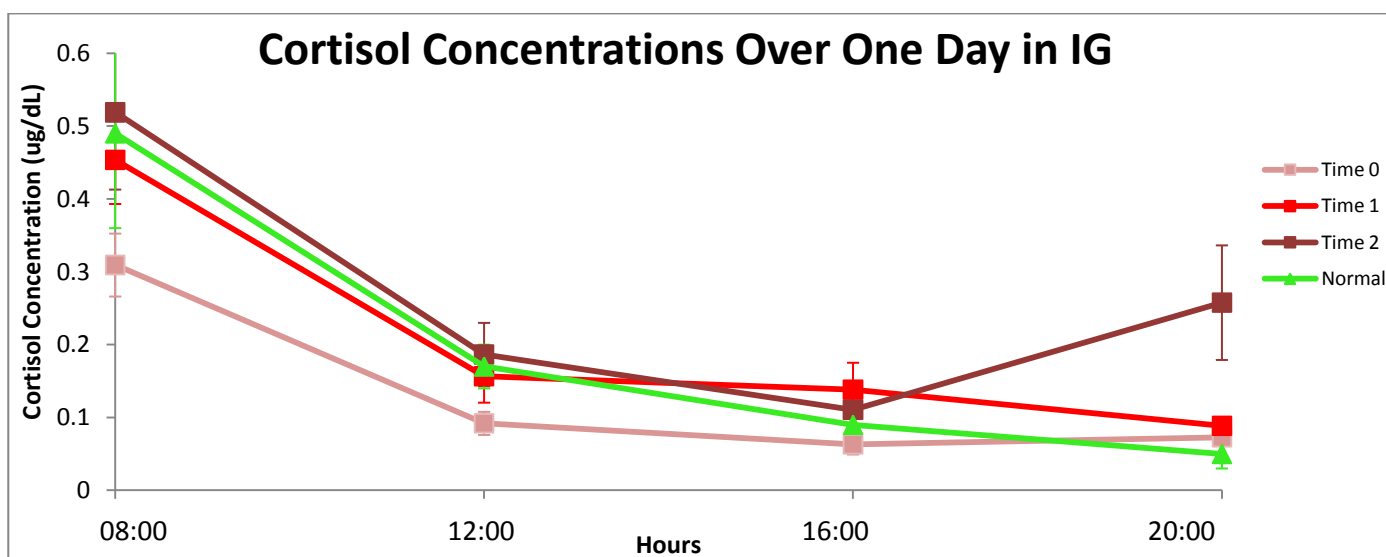


Figure 13b: Mean salivary cortisol levels of HD patients in IG at awakening, 12:00, 16:00 and 20:00h in IG at Time 0 (baseline), Time 1 (after intervention) and Time 2 (after longitudinal intervention) vs expected levels in normal population (green). Values presented as mean \pm SEM. * $P \leq 0.05$ (n)= 9.

In order to investigate net cortisol responses in patients, the sum of cortisol concentrations at each of the four time points (08:00, 12:00, 16:00 and 20:00) was calculated for each group to obtain a single net cortisol concentration for each group at each time point (Fig. 13c, Table 8).

At baseline, the sum of cortisol concentrations were not significantly different between groups ($p>0.05$) and both were below the expected range for normal individuals (HS salivary kit manual) (Fig. 13c, Table 8).

In the DIG, after the 9 month control period, cortisol levels increased by 70% to $0.81 \pm 0.16 \mu\text{g/dL}$ from baseline (shown in dotted line in Fig. 13c), an increase that was statistically significant ($p=0.007$). By contrast, after 9 months of intervention in this group, cortisol concentrations remained at the same level, $0.83 \pm 0.10 \mu\text{g/dL}$ ($p>0.05$) (Fig. 13c, Table 8).

After the first round of 9 month intervention in the IG, cortisol levels increased relative to baseline by 32% to $0.71 \pm 0.07 \mu\text{g/dL}$, and this increase was statistically significant ($p = 0.027$). After an additional 9 months of intervention (longitudinal intervention) in this group, cortisol levels increased more steeply, by 52% to $1.07 \pm 0.12 \mu\text{g/dL}$, and this rise was statistically significant ($p = 0.030$) (Fig. 13c, Table 8).

At 18 months, salivary cortisol levels were lower ($p=0.065$) in the DIG than in the IG and remained within the normative range whereas the IG had levels above the normal range (Fig. 13c, Table 8).

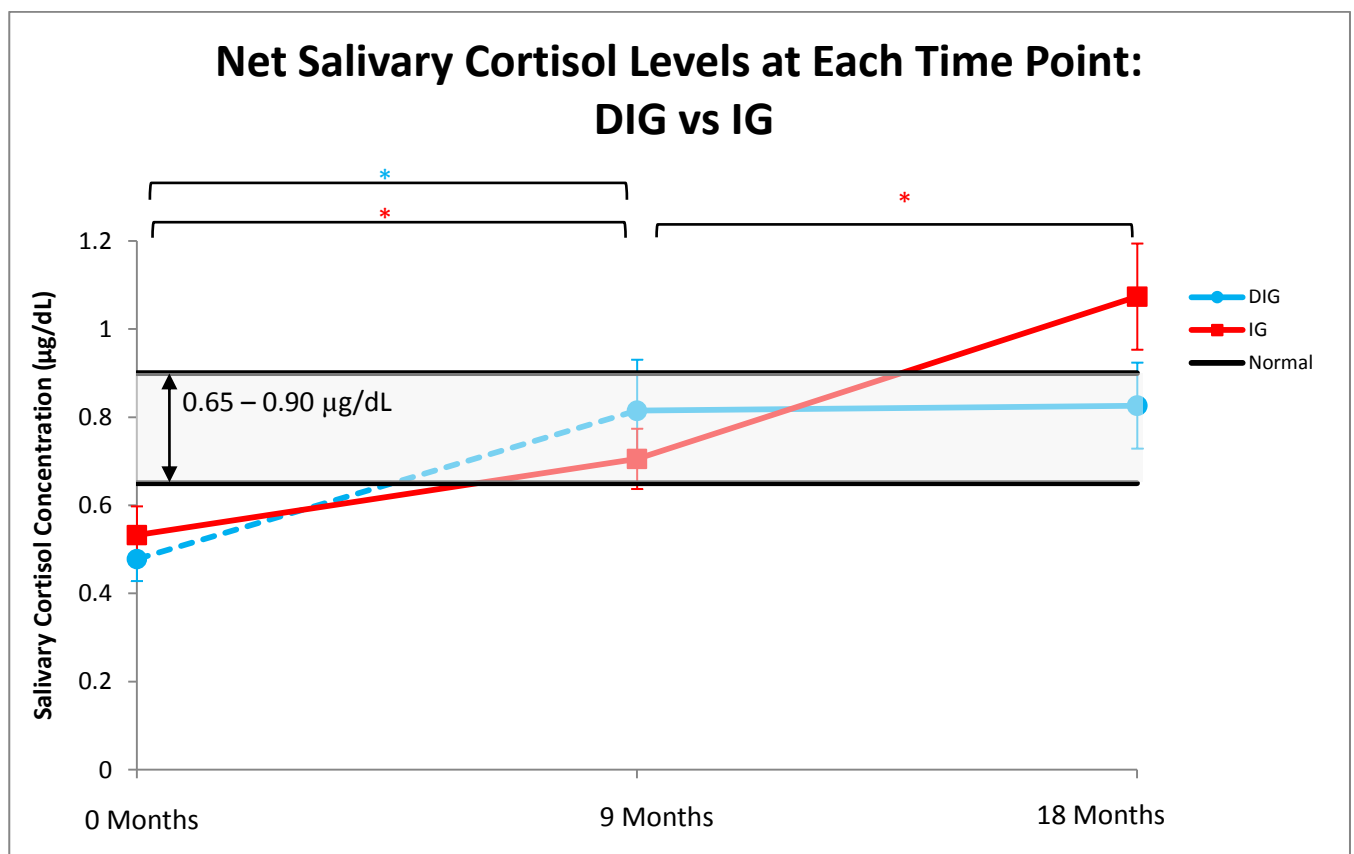


Figure 13c: The mean sum of salivary cortisol levels of HD patients at 08:00 + 12:00 + 16:00 + 20:00h at 0 Months, 9 Months and 18 Months after the commencement of the study in the DIG (blue) and the IG (red). The blue dashed line indicates the 9 month control period in the DIG. Values are presented as mean \pm SEM. * $P \leq 0.05$. DIG (n)= 12, IG (n)= 8.

In order to further investigate changes in salivary cortisol concentrations after the control period, intervention, or longitudinal intervention, the mean difference in the sum of salivary cortisol concentrations was calculated and plotted in Fig. 13d.

After the 9 month control period in the DIG, salivary cortisol levels increased by $0.34 \pm 0.11 \mu\text{g/dL}$. Conversely, after 9 months of intervention in this group, cortisol levels decreased by $0.076 \pm 0.18 \mu\text{g/dL}$ in this group, producing a significant difference between time points ($p=0.024$) (Fig. 13d, Table 8)

After the first round of 9 months of intervention in the IG, salivary cortisol levels increased by $0.12 \pm 0.11 \mu\text{g/dL}$. After the second round of intervention in this group, cortisol levels increased by a greater amount, $0.24 \pm 0.21 \mu\text{g/dL}$, however there was no statistically significant difference between the increases in cortisol between the two interventions in the IG ($p>0.05$). Salivary cortisol levels increased by an average of $0.01 \pm 0.11 \mu\text{g/dL}$ during one round of 9 months of intervention in both groups. Increases in cortisol levels were significantly less after intervention relative to the control period by a significantly lower extent in the combined intervention relative to the control period ($p=0.023$), (Fig 13d, Table 8).

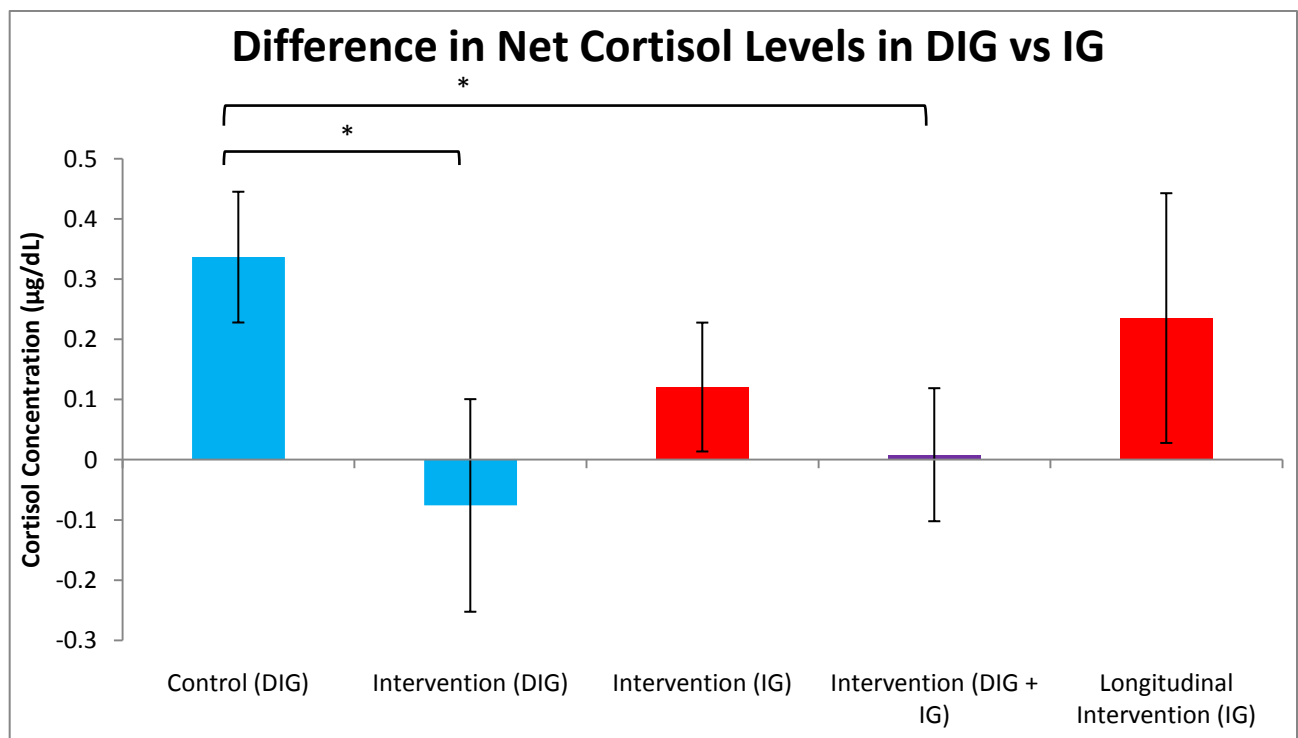


Figure 13d: The difference in the sum of salivary cortisol concentrations taken over a day for each patient was calculated between each time point and the mean of these differences after a 9 month control period in the DIG (blue), 9 months of intervention in IG (red) and the DIG (blue), after 9 months of intervention in both groups was combined (purple) and after 18 months of longitudinal intervention in the IG were plotted above. Values are presented as mean \pm SEM. * $P < 0.05$. DIG (n) = 12, IG (n) = 8.

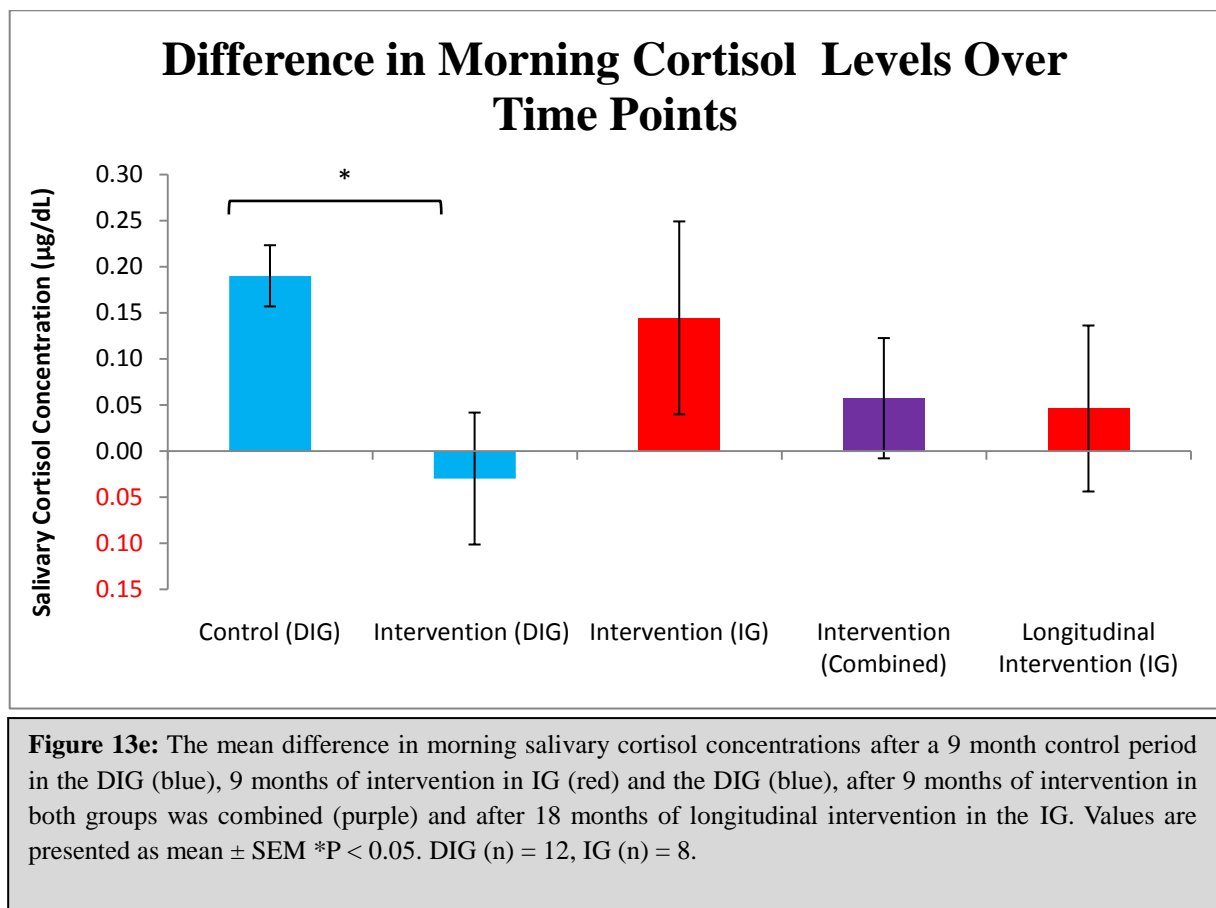
Table 8: Mean salivary cortisol concentrations at each time point in the DIG (blue) vs the IG (red) and P Values after 2-way repeated measures ANOVA analysis. Values are represented as mean \pm SEM.

	Mean Salivary Cortisol Concentration ($\mu\text{g/dL} \pm \text{SEM}$)			Mean Difference in Salivary Cortisol Levels Between Time Points ($\mu\text{g/dL} \pm \text{SEM}$)			P Value (2 Way Repeated Measures ANOVA)		
	Time 0	Time 1	Time 2	Time 1 – Time 0	Time 2 – Time 1	Time 2 – Time 0	Time 0 vs Time 1	Time 1 vs Time 2	Time 2 vs Time 0
IG	0.53 \pm 0.06	0.71 \pm 0.07	1.07 \pm 0.12	0.12 \pm 0.11	0.24 \pm 0.21	0.33 \pm 0.09	0.090	0.013	p<0.001
DIG	0.48 \pm 0.05	0.81 \pm 0.11	0.83 \pm 0.10	0.34 \pm 0.11	-0.07 \pm 0.18	0.28 \pm 0.11	0.014	0.472	p<0.001
P Value (2 Way ANOVA) IG vs DIG	0.250	0.361	0.065						

As levels of cortisol are the highest upon awakening, and changes in cortisol levels are able to be more clearly distinguished at this time point, we aimed to investigate levels in the morning in patients at different time points.

After the 9 month control period in the DIG, morning salivary cortisol levels increased by $0.19 \pm 0.03 \mu\text{g/dL}$, from baseline. Conversely, after the intervention in this group, morning cortisol levels decreased by an average by $0.03 \pm 0.07 \mu\text{g/dL}$, rendering a significant difference in the change in levels between time points ($p=0.006$) (Fig. 13e).

Following 9 months of intervention in the IG, morning cortisol levels increased by $0.144 \pm 0.11 \mu\text{g/dL}$. After the longitudinal intervention in this group, cortisol concentrations increased by $0.046 \pm 0.09 \mu\text{g/dL}$ (Fig. 13e). Although this increase was 3 fold lower than the increase seen during the first round of intervention in this group, the differences in levels between the first and second rounds of intervention were not found to be statistically significant ($p>0.05$) (Table 8).



Differences in salivary cortisol concentrations at midday (12:00h), afternoon (16:00 h) and evening (20:00h) were also analysed and although the results mimicked the pattern of change seen in morning cortisol levels, no statistically significant differences were found between time points in the DIG and IG (data not shown).

Summary of Results

Levels of C-peptide, a marker of metabolic processes in HD patients, increased throughout the study, regardless of whether patients received the intervention or underwent a control period, however increases in C-peptide appeared to be reduced by the intervention. Overall, C-peptide levels exceeded levels seen in the normal population. Cortisol, a marker of HPA function in our patients, increased after 18 months of intervention, to levels higher than normal range however, levels seemed to stabilise within the normal expected range after 9 months of optimised intervention in the DIG. BDNF, a marker of neural function, continued to decrease in patients regardless of whether they underwent a control period or 9 months of intervention, however after 18 months of intervention, levels were significantly increased in patients. There was however, a clear gender difference in the BDNF response with significantly increased BDNF levels observed only in females in response to 18 months of environmental enrichment. Eighteen months of intervention was required for BDNF levels to stabilise in males.

13. DISCUSSION

BDNF

Here for the first time, we document amelioration of BDNF deficits after longitudinal environmental enrichment in HD patients. This result is in accordance with previous studies in mice which have reported an association between environmental enrichment and the transcriptional upregulation of genes encoding neurotrophins such as BDNF, essential for neurogenesis, learning and memory (Rampon et al., 2000, Spires et al., 2004).

BDNF is the most abundant neurotrophin in the brain and is essential for neuronal survival. An enriched environment is known to enhance learning and memory performance in WT rodents (normal) (Nithianantharajah and Hannan, 2006), delay onset and cognitive decline in mouse models of HD (Pang et al., 2006) and to promote structural and morphological changes together with upregulation of BDNF expression in the brain in mouse models of HD (Spires et al., 2004, Falkenberg et al., 1992). Although these studies have been conducted in mouse models, they are in accordance with our finding of amelioration of BDNF deficits in HD patients after environmental enrichment treatment.

In this study we also demonstrate clear gender differences in the BDNF response to environmental enrichment (Fig. 11c and Fig. 11d). After 18 months of intervention, BDNF levels increased significantly in females and remained relatively stable in males (Fig. 11d). These findings are synonymous with results from previous studies which have found that responses to environmental enrichment exhibit differences with gender in mouse models of HD (Wood et al., 2010, Zajac et al., 2010). Wood et al., reported improved overall cognitive performance in female HD mouse models in comparison to male mice (2010) and increased handling of female HD mice has been reported to beneficially affect cognitive performance while having a negative effect in male mice (Wood et al., 2010). Additionally, Zajac and colleagues also demonstrated gender dependent BDNF changes in both WT mice and mouse models of HD after environmental stimuli (2010), where amelioration of BDNF deficits after wheel running were found only in female mice (Zajac et al., 2010). Similar gender-specific effects of environmental enrichment on learning and memory have been shown in mouse models of Down syndrome, where environmental enrichment had beneficial effects on spatial learning in female mice but deleterious effects in male mice (Martinez-Cue et al., 2002). This suggests that female HD patients may be more sensitive to the beneficial effects of environmental enrichment than males.

Interestingly, both genders required a longitudinal intervention of 18 months in order for improvements to take effect. Nine months of intervention did not improve BDNF levels in either gender and this result is in accordance with a previous report in which short term exercise did not induce any changes in

BDNF mRNA levels (Berchtold et al., 2010). It could be postulated that an extended period of time is required for environmental enrichment to have functional effects on HD patients.

Platelets acquire nervous system BDNF after crossing the blood brain barrier (Fujimura 2002). Hence changes in BDNF production in the brain may be reflected in BDNF concentrations in the blood. BDNF concentrations in our patients were lower than normal expected levels and this finding is in agreement with previous studies (Zuccato et al., 2001, Cattaneo et al., 2005, Ciammola et al., 2007) which have reported lowered BDNF levels in HD cells, animal models of HD and human brain tissue from HD patients. This finding is to be expected as neuronal degeneration in human HD mainly affects the striatum and cerebral cortex (Reiner et al., 1988, Rosas et al., 2008), regions where BDNF is most abundant in humans (Altar et al., 1997).

The decrease in BDNF levels could be attributed to reduced gene expression of BDNF, caused by a loss of WT HTT protein function and a gain of toxic function of the mutant HTT protein. WT HTT stimulates BDNF gene transcription by acting at the level of the BDNF exon 2 promoter whereas the presence of the pathological CAG expansion in HD abolishes the ability to sustain BDNF gene transcription in HD (Zuccato et al., 2001). Overexpression of WT HTT has been reported to increase BDNF production (Zuccato et al., 2001); And it has been found that BDNF exon 2 promoter is 60% less active in cells over expressing mutant HTT than in parental cells (Zuccato et al., 2001). Reports have found that the WT, but not the mutant, protein influences the activity of a repressor element 1/neuron-restrictive silencer factor (RE1/NRSE) within the BDNF exon 2 promoter (Zuccato et al., 2001). In addition to decreased BDNF exon 2 promoter activity in HD, BDNF exon 3 and 4 have also been indicated in HD and reduced BDNF mRNA 3 and 4 has been reported in animal models of HD (Hermel et al., 2004).

In future studies it would be interesting to analyse if the impaired BDNF anterograde transport, which has been extensively reported in HD, is influenced by environmental enrichment. This was not possible by analysing peripheral levels of BDNF in our study, however previous studies in mouse models of HD have found that environmental enrichment rescues striatal and hippocampal protein deficits whereas antero-medial cortical levels of BDNF are unaffected (Spires et al., 2004). This could mean that environmental enrichment rescues the impaired cortico-striatal transport of BDNF. MRI studies of environmentally enriched HD patients are currently underway and will provide information on rescue of grey matter which could contribute to rescue of BDNF levels. Furthermore, studies analysing post mortem brain tissue in environmentally enriched HD patients compared to control HD patients mapping the localisation of BDNF would be of interest.

BDNF deficits contribute significantly to the pathogenesis of HD (Canals et al., 2004). Mouse models of HD which were crossed with heterozygous BDNF knock-out mice showed an earlier onset of motor dysfunction, reduction of striatal volume attributed to a loss of striatal neurons which suggests that it may be a valuable candidate for therapeutic intervention in HD (Canals et al., 2004). Studies in which BDNF has been delivered by lentiviral transfection to primary neurons expressing mutant HTT have demonstrated that it protects against cell death induced by the mutant HTT (Canals et al., 2004). Despite these promising results, exogenous BDNF supplementation raises a number of different problems, the first of which is determining the amount of BDNF that is required to reach the affected neurons, which is compounded by the fact that BDNF is relatively unstable and only a small amount is able to cross the blood brain barrier (Knusel et al., 1992, Pardridge, 2002). If the amount of BDNF reaching the affected neurons is too small it may not be sufficient to produce the required effects. In contrast if the amount is too large, it may be deleterious as high BDNF levels have been shown to down regulate the expression of the BDNF receptor, tyrosine-related kinase B receptor, thus reducing the signalling pathway activated by BDNF and therefore blocking any beneficial effect (Frank et al., 1996, Knusel et al., 1997, Sommerfeld et al., 2000). Moreover, excess BDNF has been reported to give rise to epileptic activity (Binder et al., 2001). It could be because of these reasons that subcutaneous and intrathecal BDNF administrations have met with little success (1999, Ochs et al., 2000). Environmental enrichment and physical exercise, therapies which have been observed to enhance endogenous production of BDNF have recently become of increased interest as they provide BDNF levels in normal physiological quantities.

As BDNF is also produced by muscle cells during contraction (Pedersen et al., 2009), it could be argued that our BDNF measurements do not reflect the in-brain concentration of this neurotrophic factor, and that the exercise undertaken by our patients may have caused a transient increase in muscle-derived BDNF. It has been reported however, that BDNF produced by muscle cells during contraction exerts its actions locally and is not released into the circulation (Pedersen et al., 2009). In addition to expression in skeletal muscle and CNS, BDNF has also been shown to be expressed in liver (Cassiman et al., 2001) heart, lung (Timmusk et al., 1993), pancreas (Hanyu et al., 2003) and spleen (Laurenzi et al., 1994). Because our ELISA kit detects all isoforms of BDNF, it could be suggested that the peripheral BDNF changes observed in our patients' whole blood were reflective of changes in BDNF expression from sources other than brain tissue. However, a recent study has confirmed a positive correlation between whole blood BDNF levels and hippocampal levels in rats (Klein et al., 2011). It would be useful to investigate if this correlation is replicated in humans.

Antidepressants, namely selective serotonin reuptake inhibitors (SSRIs), lithium, memantine, riluzole, cystamine and cysteamine have all been reported to increase BDNF levels (Chuang, 2004). Because depression is a common feature of HD, a number of patients who were involved in the study were prescribed anti-depressants. Because all patients in the study remained on the same dosage of anti-depressant medication throughout the study, anti-depressant use was not accounted for during analyses of BDNF results.

Alcohol intake has also been reported to influence BDNF levels (McGough et al., 2004). Frequency and amount of alcohol consumption was not recorded in our study, however in future studies, it would be interesting to examine the effect of alcohol consumption on BDNF levels in HD patients.

Platelets are one of the major BDNF storage sites in the periphery in human beings (Fujimura et al., 2002), however no association between BDNF and thrombocyte count has been reported (Trajkovska et al., 2007) therefore platelet count was not measured in our patients. Aspirin treatment has been reported to not affect platelet count (Erhart et al., 1999) furthermore, aspirin does not affect the release of BDNF from platelets (Stoll et al., 2011). It is for these reasons that patients intake of aspirin was not accounted for in BDNF analyses.

In order to further solidify our findings, it would be interesting to assess levels of BDNF after a period of detraining in our patients.

It would be interesting to analyse other biochemical markers in the brain as markers of neurogenesis including fibroblast growth factor (FGF). FGF is angiogenic, stimulates proliferation of astrocytes and promotes survival and growth of neurons and has been reported to be increased by exercise (Gospodarowicz et al., 1987). Moreover, epidermal growth factor (EGF) is important in promoting survival and differentiation of post mitotic neurons and increases the density of newborn cells in the subventricular zone. It would also be interesting to investigate glutamatergic activity which has been reported to increase after exercise (Poon et al., 2006). Glutamate is involved in the mechanisms that promote learning and memory and activation of glutamate receptors has a role in the generation of long term potentiation as a response to exercise (Holscher et al., 1999). Glutamate receptors, GluR1 and GluR2, are related to long term potentiation mechanisms and undergo plastic changes after exercise in healthy individuals (Dietrich et al., 2005, Real et al., 2010).

C-peptide

Insulin levels continually increased throughout the 18 months of the study and exceeded normal expected values (Fig. 12a). This result is in contrast to previous reports of impaired insulin secretion and decreased insulin gene expression in mouse models of HD (Andreassen et al., 2002, Hurlbert et al., 1999, Bjorkqvist et al., 2006). These studies were carried out in mouse models of HD which expressed a highly expanded CAG repeat (145-150 CAG repeats), much longer than the average CAG repeat length observed in our patients (43.4 CAG repeats). It is known that a correlation between CAG repeat and glucose homeostasis exists, and glucose responsiveness decreasing with increased CAG repeat has been reported (Josefsen et al., 2008). It would therefore be interesting to investigate if glucose homeostasis abnormalities are observed in mouse models of HD expressing mutant HTT with a repeat length within the range normally found in HD patients.

A study conducted in Serbia in 2008, (Lalic et al., 2008) reported impaired first phase insulin secretion in HD patients. Comparisons of the characteristics of the group of patients in the study by Lalic and colleagues (Lalic et al., 2008) with the patients in our study, reveals stark differences in the mean disease duration between groups (7.1 years in the Lalic study versus 3.35 years in our study) which suggests that impairments in insulin secretion may be evident with greater progression of disease. Moreover, it is possible that the differences in the genetic background between the two groups could contribute to the differences in insulin secretion.

Our results are also confounded by findings in earlier studies which have reported increased prevalence of diabetes in HD patients, (Podolsky and Leopold, 1977, Farrer, 1985, Schubotz et al., 1976), none of our patients were reported as diabetic. The difference in findings could be attributed to the fact that these studies were all carried out before a genetic test for HD became available and the patients are therefore not likely to be well characterised.

Insulin levels in our patients exceeded the normal expected range in both the DIG and IG, which may indicate the presence of impaired insulin sensitivity. Decreased insulin sensitivity has been reported in HD patients and mouse models of HD (Lalic et al., 2008, Bjorkqvist et al., 2006). Intranuclear inclusions formed from aggregates of the mutant HTT protein have been found to be present in pancreatic cells (Sathasivam et al., 1999, Andreassen et al., 2002). Therefore it is postulated that intranuclear inclusions in pancreatic cells of HD patients may contribute to alterations in insulin feedback mechanisms and impairments in beta cell function. Future tests that analyse insulin sensitivity and beta cell function such as a homeostatic model assessment (HOMA) would be useful in helping to determine if the elevated insulin levels observed in our patients are a result of impaired insulin sensitivity.

A decrease in the elevation of insulin was seen after intervention compared to the control period in the DIG, which may suggest that the enrichment program was partially alleviating the elevated levels of insulin. Physical exercise has been widely reported to improve insulin sensitivity and improve glucose homeostasis in healthy individuals (van der Heijden et al., 2009, Gordon et al., 2012, Maarbjerg et al., 2011) and this slight decrease in elevation of insulin could be attributed to the enrichment undertaken by the patients.

Finally, it could be speculated that a portion of the rise in C-peptide levels in our study could be a result of the progression of the disease through time, as to the best of our knowledge this is the first longitudinal study which has analysed C-peptide levels in HD patients. Future studies with a larger sample size are warranted to confirm this hypothesis. From our results, it can be concluded that there is a significant fluctuation in insulin levels over the 18 months of the study, and testing insulin sensitivity of patients could be informative in future studies.

CORTISOL

Throughout the study, cortisol levels continued to increase in patients in the IG group and exceeded normal expected levels. They also increased during the control period in the DIG (Fig. 13c). This finding is in line with previous studies that have assessed cortisol levels in HD patients and have reported elevated cortisol production rates (Heuser et al., 1991, Leblhuber, 1993, Aziz et al., 2009b). This finding could indicate HPA axis dysfunction and suggests that a disturbed central glucocorticoid feedback may be present in HD patients (Aziz et al., 2009b).

Intranuclear inclusions containing the mutant HTT protein are evident in HD and substantial hypothalamic atrophy and cell loss have been reported in HD patients (Kassubek et al., 2005). The reason why the hypothalamus is preferentially affected in HD is unknown, however it has been postulated that it could be as a result of increased interaction of mutant HTT with huntingtin-associated protein 1 (Hap1) which is more abundantly expressed in the hypothalamus than in other brain regions (Sheng et al., 2006). This could be related to the dysfunction of the HPA axis as evidenced by the finding of abnormal levels of cortisol in the IG and increasing levels during the control period in the DIG (Fig. 13c).

Moreover, impaired glucocorticoid receptor function in the hypothalamus could underlie the elevated cortisol levels in patients (Aziz et al., 2007). The negative feedback mechanism of cortisol production is regulated by two receptor subtypes in the brain; High affinity mineralocorticoid receptors in the hippocampus, which determine basal cortisol levels, and low affinity glucocorticoid receptors in the hypothalamus, particularly in the paraventricular nucleus. Paraventricular nucleus pathology has been

reported in HD and is likely to contribute to the altered HPA axis seen in our patients (Aziz et al., 2007, Vogt and Vogt, 1952). Additionally, as with BDNF, the mutant HTT protein may contribute to alterations in gene expression of cortisol, however this hasn't been studied in HD yet. It would be useful to employ a dexamethasone suppression test in order to more fully assess feedback mechanisms within the HPA axis in HD patients.

An altered immune profile is evident in HD patients, and neuroinflammation in HD has been reported (Moller, 2010, Crocker et al., 2006). Elevated levels of inflammatory mediators including IL-1b and TNF- α have been detected in the cerebrospinal fluid and plasma of HD patients (Moller, 2010). Cortisol is one of the most potent anti-inflammatory hormones in the body (Cupps and Fauci, 1982) therefore it could be postulated that the elevated levels of cortisol observed in our patients are in response to the elevated inflammatory mediators present in HD patients.

The anti-inflammatory effects of exercise have been widely reported and are dependent upon the frequency and amount of exercise carried out (Gleeson et al., 2011). Cortisol levels stabilised after nine months of optimised intervention in the DIG (Fig. 13c) which comprised of three-times-weekly exercise. However cortisol levels did not stabilise after eighteen months of a sub-optimal intervention in the IG. Therefore it is possible that neuroinflammation in HD patients is partly alleviated by the optimised intervention, which in turn leads to alleviation in cortisol levels. However this remains speculative at this stage and may be worthy of investigation in future studies.

The optimised intervention undertaken by the DIG for nine months was comprised of 3-times-weekly supervised intervention. Increased social interaction has been reported to ameliorate depressive symptoms (Drevets, 2001) and to reduce cortisol levels (Uvnas-Moberg, 1998). Therefore it is suggested that the social stimulation associated with the supervised intervention contributed to the alleviation of cortisol levels during the 9-18 month period in this group.

It must be noted that medication records of patients indicated that no patients were prescribed glucocorticoid medication such as prednisone and prednisolone. Androgens and phenytoin have been reported to decrease cortisol measurements (Abraham et al., 1976), however, none of our patients were on these medications.

Analysis of results from quality of life questionnaires filled out by the patients is currently underway and therefore cannot be commented on. It would be interesting to determine if a correlation exists between patients' cortisol measurements and their perception of quality of life.

Even mildly elevated cortisol levels have been associated with a number of clinically significant health effects such as cognitive dysfunction (Lupien et al., 1998), plasma lipid disturbances, low bone density (Raff et al., 1999), and increased morbidity related to cardiovascular disease (Otte et al., 2004), insulin resistance and major depression (Brown et al., 2004). HD patients exhibit a number of symptoms and signs that could be partly attributed to hypercortisolism such as memory deficits, mood disturbances, skeletal muscle atrophy, impaired glucose tolerance, and decreased hippocampal volume. The speculated impairment in insulin sensitivity observed in our patients in this study could partly be attributed to elevated cortisol levels in our patients. Larger scale studies however, are needed to confirm an association between high cortisol levels and clinical phenotype in HD patients. These findings are of importance as anti-glucocorticoid therapy is presented as a therapeutic potential for HD patients and environmental enrichment has been demonstrated as being a potential therapy of alleviating hypercortisolism in HD patients.

13.1 LIMITATIONS

Due to the low prevalence rate of HD, our sample size is small (n=20) and this presented as the biggest limitation as it decreased the statistical sensitivity of our study. In order to minimise this limitation, appropriate stringent statistical analyses were conducted, such as a two way ANOVA to assess the significance of our results. Although we observed substantial changes in biochemical markers between time points, in some cases, they were considered statistically insignificant. We predict that with a larger sample size statistical significance will be attained. Although it could be speculated that patient compliance was a limiting factor in our study, we had greater than 80% compliance for all aspects of the study.

14. CONCLUSIONS

HD is a devastating disease and current pharmacological therapies do nothing to delay onset but act to alleviate symptoms in patients. Environmental enrichment has been shown to be successful in delaying onset and progression in several neurodegenerative diseases including Parkinson's and Alzheimer's disease, and short trials have shown promising results in HD patients. In this study we proposed to study the effects of an extended trial of environmental enrichment intervention on physiological processes by analysing biochemical markers indicative of neural, metabolic and neuroendocrine pathways. We showed that environmental enrichment produced positive benefits even with a small sample size. The results from this preliminary study will inform the scientific community of the positive effects of environmental enrichment on HD patients, in order for studies with a larger sample size to take place in the hope that environmental enrichment will be integrated into current HD therapies.

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